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RENAL ASPECTS OF EXPERIMENTAL AND CLINICAL HYPERTENSION*

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ESSENTIAL and malignant hypertension were separated from the renal hypertension of Bright's disease by the accurate clinical and pathologic observations of Allbutt, Janeway, Volhard, Keith, and others. The intuitive concept of a renal origin of all, or at least of most, syndromes of increased arterial pressure was discarded because of (a) the manifest differences in clinical course which separate Bright's disease from essential and malignant hypertension; (b) the characteristic differences of degree and kind of renal structural change in nephritis and hypertension; (c) the view that, in early hypertension, the kidney is structurally and functionally intact and that, therefore, it cannot be the origin of the disease.

While the first and second of these criteria are undoubtedly true, they do not in themselves establish the extrarenal origin of essential and malignant hypertension. The third criterion, which supposes the integrity of the kidney in hypertension, seems to be invalidated by recent observations on the structure and function of the kidney in hypertension. Since the supposition of an extrarenal pathogenesis of essential and malignant hypertension largely depends on this assumption, a renal origin of hypertension is, therefore, not excluded.

Goldblatt, Lynch, Hanzal, and Summerville[1] have established that a syndrome similar in many ways to essential and malignant hypertension in man may be produced experimentally in animals as the result of partial compression of one or both main renal arteries. Experimental hypertension of apparently identical character has also been obtained by compression of the renal parenchyma in the fibrocollagenous scar of perinephritis due to application of silk or cellophane.[2] This development of experimental counterparts of hypertension

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has prompted an upsurge of interest in the relation of the kidney to increased arterial pressure, which has led to new concepts of the pathogenesis and clinical physiology of both the experimental and clinical disease. These concepts and methods in which they find their application form the subject of this review.

ORIGIN OF EXPERIMENTAL HYPERTENSION

Hypertension produced by compression of the renal arteries or of the renal parenchyma has been widely regarded as the result of renal ischemia. In particular, it is attributed to ischemia of functional renal tissue, since widely devitalized kidneys do not function as sites of origin of experimental hypertension.[3] Measurements of renal blood flow in uninephrectomized dogs made hypertensive by compression of the renal artery have shown a decrease which averaged 40 per cent and ranged from 18 to 63 per cent.[4] Further, it has been widely observed that experimental hypertension is more severe and more often malignant when renal arterial occlusion is nearly complete.[5]

Some doubt was cast on the belief that renal ischemia as such is the cause of experimental hypertension by the observation that urea clearance was sometimes normal in experimentally hypertensive animals,[6] since urea clearance in normal dogs parallels the rate of renal blood flow.[7] The further observation that the renal clearances of phenol red, inulin, and creatinine were in some instances not altered during the induction or course of experimental hypertension added weight to this doubt.[8] Indirect measurements of renal blood flow before and after compression of the renal artery in uninephrectomized dogs with single explanted kidneys have since shown that hypertension of moderate degree may be induced without any significant or lasting change in renal blood flow (Chart 1), while in some instances renal blood flow may be reduced without the occurrence of an increase in arterial pressure.[9]

The apparent paradox that compression of the renal artery sufficient to induce changes in the kidney which lead to hypertension need not always be associated with renal ischemia demands an explanation that depends upon a consideration of the mechanism of blood flow. Thus, it should be noted that the rate of blood flow is the product of *mean* arterial pressure and of peripheral resistance. If, with increased resistance, there is a corresponding increase of pressure, no reduction of flow need occur. In any case, a high degree of arterial occlusion is necessary in order to reduce mean arterial pressure and blood flow distal to the point of compression.[10] It should also be observed that the initial effect of arterial compression is a reduction of pulse pressure distal to the point of clamping, while mean arterial pressure and, therefore, blood flow, remain unchanged. It follows from these principles that arterial compression, as by the Goldblatt clamp, will nearly always result in a decrease of pulse pressure distal to the clamp and, if the constriction is sufficiently intense, will be associated with a decrease of mean pressure and blood flow. However, if mean arterial pressure proximal to the clamp is simultaneously increased, and if the compression is not excessive, neither mean arterial pressure nor renal blood flow need be reduced by clamping.

The view that renal hypertension may occur in the absence of renal ischemia is supported by the observation that mean renal arterial pressure in some experi-

mentally hypertensive dogs is normal distal to the point of clamping.[11] It is at least unlikely that renal blood flow would be affected by clamping when mean arterial pressure was not. Of interest also are observations[12] which demonstrate that, following clamping of the renal artery, there is a tendency for renal blood flow, which initially had decreased, to return to, or toward, normal levels.

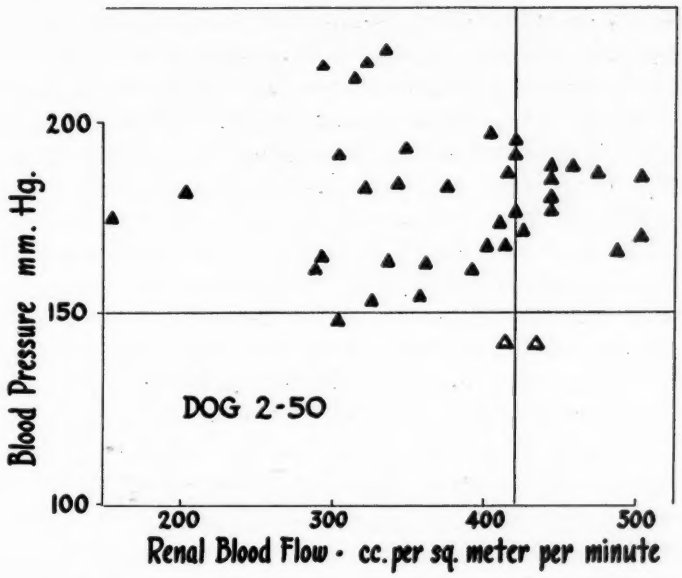

Chart 1.—Relation of mean femoral arterial pressure to total renal blood flow[11] in an uninephrectomized dog before (△) and after (▲) application of a Goldblatt clamp to the main renal artery. The wide variations in blood flow over a period of more than one year are accounted for by repeated adjustments of the degree of arterial constriction. Normal blood flow persisted during moderate hypertension.

If then, experimental renal hypertension may persist in the absence of renal ischemia, the decreased pulse pressure which follows clamping remains as a possible cause of the renal change that leads to hypertension. Experiments were, therefore, performed in which isolated dog's kidneys were perfused with blood under conditions which were as physiologic as possible. A clamp was gradually adjusted on the renal arteries to a point where pulse pressure was greatly reduced while renal blood flow was either unaffected or slightly decreased. Arterial pressure was then increased proximal to the compression so that both mean arterial pressure in the renal artery and renal blood flow remained within normal limits. Under these circumstances, the perfused kidney liberated renin, the so-called renal pressor substance, into the renal venous blood.[13]

It appears, therefore, that renal ischemia is not the necessary cause of renal hypertension, and the suggestion is made that intrarenal reduction of pulse pressure may be the exciting cause. However, it should be noted that those circumstances which excite experimental renal hypertension, possibly by reduction of pulse pressure, also, when applied with sufficient intensity, result in reduction of renal blood flow, so that the level of renal blood flow in experimental hypertension depends upon a balance struck between increased arterial pressure and increased renal arterial resistance.

The mechanism by which reduction of pulse pressure may lead to the establishment of hypertension is not clear. It is known that organs perfused in the absence of pulsatile flow speedily become edematous, and that their cells may lose their normal permeabilities to vital dyes.[14] It is, therefore, conceivable that renal cells are normally impermeable to renin; this is evidenced by the absence of renin from normal renal venous blood. But reduction of pulse pressure increases renal cellular permeability so that renin escapes; this is supported by the presence of renin in the renal venous blood of hypertensive animals and man.[15]

If pulse pressure rather than blood flow is the determinant in the liberation of renin, it follows that renin must arise in some site normally exposed to a high pulse pressure, i.e., proximal to the glomerulus, and, probably, in an area in close relation to the afferent arteriole. Modification of the muscle cells of the afferent arterioles into larger cells which group around the glomerular vascular pole have been described (reviewed by Smith[16]). Although disagreement exists as to the character and normal distribution of these cells, they are regarded by some as of epithelioid nature and are stated to contain fuchsinophilic granules. Largely on the basis that these cells are hypertrophied and their granules more numerous in experimental and clinical states associated with hypertension,[17, 18] it has been suggested that this "juxta-glomerular-myofibrillary organ" is the site of renin formation.[19] Although the evidence which supports this view is entirely circumstantial, it is at least noteworthy that the position of these cells in the kidney is such that they are exposed to the extremes of pressure which occur in the afferent arterioles.

RENAL PRESSOR SYSTEM

It has been assumed in part of the above discussion that the liberation of renin somehow leads to the establishment of experimental and, possibly, of clinical hypertension. This association of renin to hypertension must now be examined.

The existence of renin, a proteinlike pressor substance in renal cortex, was first observed in 1898.[20] Various aspects of the original study have been confirmed and extended, largely as an aspect of the recent renewal of interest in renal hypertension. Methods of purification of renin have been described,[21-23] since crude extracts of kidney are contaminated by depressor substances.

It was observed that purer fractions of renin had little or no effect on the blood vessels of isolated organs (dog's tail, rabbit's ear) perfused with Ringer's solution,[24] while the addition of blood, plasma, or pseudoglobulins of plasma to renin in Ringer's solution resulted in prompt vasoconstriction. It was concluded that the pseudoglobulins of plasma contain a substance tentatively connoted renin activator. The activation of renin was then shown to be due to the interaction of renin and renin activator, with a resultant liberation of a third substance, angiotonin.[25] Angiotonin, unlike renin and renin activator, is not a protein. It is thermostabile and dialyzable, and certain of its crystalline derivatives have been prepared. Its action as a pressor substance is both prompt and of short duration, and is, therefore, unlike the slower rise and sustained increase of arterial pressure which follow injections of renin.

Repeated injections of renin into normal animals result in a loss of pressor responsiveness (tachyphylaxis) which, in part, is due to a loss of activator by

combination with the injected renin. However, restoration of activator by transfusion or injection of activator concentrates does not fully restore the pressor action of renin. Repeated injection of angiotonin causes loss of responsiveness also, but this tachyphylaxis does not develop as rapidly as that due to injection of renin and plasma transfusion, and injection of activator does not affect it. Bilaterally nephrectomized animals are sensitized to the pressor action of renin and angiotonin and do not develop tachyphylaxis to angiotonin and only slowly to renin. Angiotonin tachyphylaxis and the residual inhibition of the pressor action of renin after restoration of activator, therefore, seemed to be due to the liberation of an angiotonin inhibitor from the kidneys.[26]

Harrison, Grollman, and Williams,[27] and one of us and co-workers[28] have been successful in attempts to extract such an inhibitor or antipressor substance from the kidneys. The evidence at present available indicates that we are dealing with the same active principle as that reported shortly before by Harrison, Grollman, and Williams. Our work was based on different reasoning and was done independently. The two groups are now collaborating toward a further solution of the problem. The in vitro activity of such extracts on angiotonin (hypertensine) has recently been reported.[29]

To summarize, the pressor system may be considered as originating in renin which, when released into the blood, combines with renin activator to yield angiotonin. Angiotonin acts as the effective vasoconstrictor and pressor agent. Opposing or balancing the action of angiotonin, the kidney contains a substance which acts as an inhibitor of the action of angiotonin. The relation of this pressor system to experimental and clinical hypertension is suggested by the following observations: (a) renin is present in the renal venous blood of hypertensive but not of normal animals; (b) the blood of hypertensive animals and human beings has properties which suggest that it contains less angiotonin inhibitor than normal blood; (c) nephrectomized and, possibly, hypertensive animals are more sensitive to the pressor action of angiotonin than are normal animals; (d) the peripheral blood of hypertensive animals and human beings contains a vasoconstrictor which, like angiotonin, is potentiated in the presence of blood from nephrectomized animals[30]; (e) as will be discussed below, the nature of the pressor action of angiotonin is not inconsistent with that of a substance which might participate in the genesis of hypertension.

Independent and almost simultaneous confirmation of this concept of the humoral pressor system in hypertension is given by the findings of Braun-Menendez, Fasciolo, Leloir, Muñoz, Taquini, and Houssay, of Buenos Aires.[31] These workers have also shown that a pressor substance, apparently renin, is liberated from ischemic kidneys and, proceeding from the observation that renin is not in itself vasoactive,[24a] have shown that it interacts with a pseudoglobulin, renin activator (called by them hypertensinogen), with liberation of an effector substance, similar in physiologic properties to angiotonin, to which they refer as hypertensine. The chemical similarity or identity of angiotonin and hypertensine remains to be established. Indirect evidence has led them to the conclusion that there exists in normal kidneys an angiotonin inhibitor, called by them hypertensinase, since it is their view that it may act by enzymatic destruction of hypertensine (angiotonin).

Other pressor substances have been described as originating in the kidney. Thus, "perfusin" which, unlike renin and angiotonin, is potentiated by cocaine, has been obtained by perfusion of the kidneys of normal hogs and dogs with Ringer's solution.[32] A substance, whose properties are not unlike those of renin, is found in the renal venous blood of *completely* ischemic kidneys and has been provisionally called ischemin.[33a] It now appears that this substance is renin.[33b] Also interesting in this connection is the observation that isolated ischemic kidneys or anaerobic kidney tissue *in vitro*,[34a] as well as completely and partially ischemic kidneys *in vivo*,[34b] form a pressor substance, apparently hydroxytyramine, from "dopa" (dihydroxyphenylalanine). This substance, like "perfusin," is potentiated by cocaine and may be similar to the pressor substance formed during the anaerobic incubation of kidney tissue.[35] These observations are of particular interest in view of the reported activity of tyrosinase in restoring to normal increased arterial pressure in hypertensive rats and dogs.[36] It should, however, be observed that formation of apparent hydroxytyramine is largely restricted to conditions of anaerobiosis and extreme oxygen want, whereas the oxygen content of renal venous blood in hypertensive dogs, even in the presence of moderate renal ischemia, is within normal limits.[4, 11] It is doubtful whether anaerobic conditions could prevail in kidneys while the oxygen content of the blood remains unchanged.

RENAL FUNCTION IN HYPERTENSION

The supposed integrity of the kidney in hypertension was based largely on the absence of proteinuria and hyposthenuria and on the normal urea clearance of many hypertensives, since it was assumed that an abnormality of renal function would be revealed in one or the other of these tests. Unfortunately, hyposthenuria, when present in mild degree, may not manifest itself unless efforts are made to obtain urine of maximum specific gravity, as in the Addis concentration test, and, when such efforts are made, it can be shown that the majority of hypertensives whose urea clearance is normal show definite impairment of the maximum ability to concentrate urine (Chart 2). More recently developed methods of study of renal function have shown that even in early cases of hypertension there is evidence of some degree of renal ischemia, apparently due to constriction of the glomerular efferent arterioles. These methods, the determination of the clearances of diodrast, phenol red, and inulin, and of the maximum ability of the kidneys to secrete diodrast and reabsorb glucose, have been developed and widely applied by Smith, Shannon, Goldring, Chasis, Ranges, and their co-workers.[16, 37]

Briefly, inulin is excreted only by glomerular filtration. Its clearance (volume of plasma equivalent to the amount of inulin which appears in the urine each minute) is, therefore, equal to the volume of glomerular filtrate formed each minute. Diodrast, on the other hand, is excreted largely by tubular secretion, i.e., by transfer of diodrast from the plasma of the peritubular capillaries across the cells of the proximal convoluted tubules into the tubule lumen. This removal of diodrast from blood in the kidney is nearly complete at low plasma concentrations of diodrast, i.e., when diodrast is excreted at less than a maximum rate. If renal diodrast load is large, so that more is present in the kidney than

can be transferred by its cells, the removal of diodrast from blood then becomes less complete, since excretion is already at a maximum. However, since at low diodrast loads extraction approaches completion (80 to 90 per cent), it follows that diodrast clearance at such loads is nearly equivalent to renal plasma flow, or, at least, to the rate of flow of plasma to functioning tubular areas (effective renal plasma flow). It may be assumed that the proportion of diodrast left in the renal blood at low renal loads (10 to 15 per cent of that present in arterial

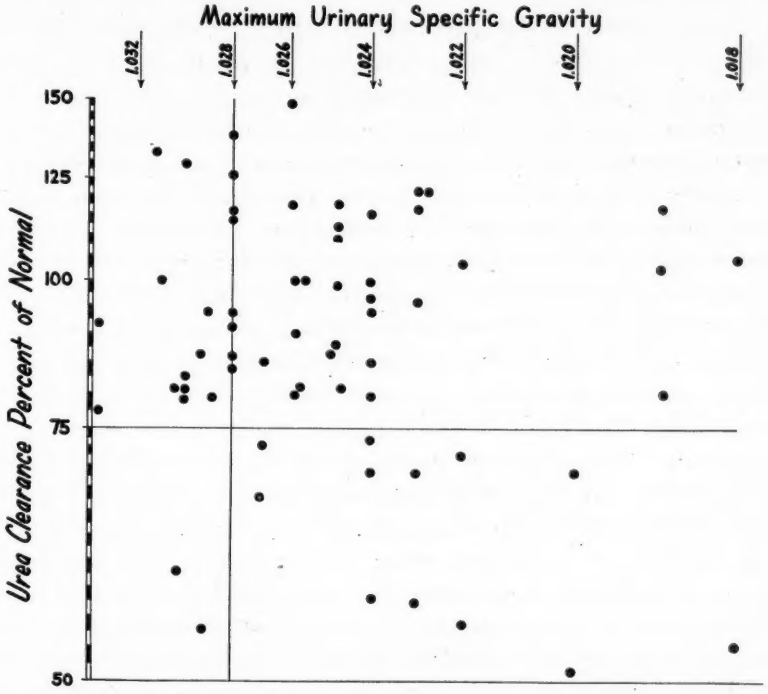

Chart 2.—Relation of urea clearance and maximum urinary specific gravity in 72 cases of essential hypertension. Abscissa (urinary specific gravity by Addis test) and ordinate (urea clearance) are plotted logarithmically. Maximum urinary specific gravity is within normal limits in only 22 instances, while normal values of urea clearance were observed in 50 patients.

blood) represents that volume of total renal blood flow which traverses supporting and nonexcreting renal tissues. A small proportion of the urinary diodrast is obtained from the red blood cells, so that it is inaccurate to assume that urinary diodrast is wholly derived from plasma. For practical purposes, since the proportion of diodrast added from the red blood cells is equivalent only to from 10 to 15 per cent of the diodrast excreted, it may be assumed that apparent plasma diodrast clearance is nearly equivalent to total renal plasma flow.[38, 39] It follows that renal blood flow may be calculated from plasma diodrast clearance if the fraction of plasma present in the blood (1-hematocrit index) is known. Normal levels of diodrast clearance, renal blood flow, inulin clearance, and ability to excrete diodrast in man have been established.[40]

Phenol red is excreted by a mechanism similar to that of diodrast, but, apparently, less efficient, since only about 50 per cent of the phenol red is removed from the blood.[41] Phenol red clearance is, therefore, equivalent to roughly 50

per cent of renal plasma flow, and, in man, to about 56 per cent of plasma diodrast clearance, viz., roughly 375 c.c. per minute per 1.73 square meters of body surface.

Simultaneous measurement of inulin and diodrast clearances yield values equivalent, respectively, to the rate of glomerular filtration, i.e., the volume of water removed from the plasma by filtration; and to the volume of plasma from which the filtrate was formed. Increased intraglomerular pressure increases the proportion of water squeezed from the plasma through the glomerular capillaries, and thus increases the ratio of $\frac{\text{inulin}}{\text{diodrast}}$ clearance (filtration fraction). This ratio, which represents the fraction of plasma water filtered, is, therefore, a measure of changes of intraglomerular pressure. In hypertension, diodrast clearance, i.e., renal plasma flow, is usually decreased below normal levels, while inulin clearance is maintained. The normal level of inulin clearance depends upon increased intraglomerular pressure. The increased intraglomerular pressure of hypertension is due neither to the high level of systemic arterial pressure nor to their dilatation of the afferent arteriole, since either of these changes would of themselves increase renal blood flow, which, in hypertension, is decreased. It follows that both the decreased renal plasma flow and the maintenance of filtration rate in hypertension are the result of constriction, predominantly of the glomerular efferent arterioles.[42]

The increased filtration fraction of hypertensives maintains glomerular filtration within normal limits long after there has occurred a marked decrease in renal blood flow, and thus explains the maintenance of normal urea clearance until late in hypertension, since urea is also excreted by glomerular filtration. This fact also may explain the observation that concentrating power usually fails before urea clearance decreases, since the concentration of urine depends upon the capacity of the renal cells to work, and it may well be that the ischemic cells of hypertensives are unable to work efficiently. The maintenance of glomerular filtration also implies that recourse to measurement of the blood levels or clearances of the other substances excreted by filtration (nonprotein nitrogen : urea nitrogen ratio, sucrose, creatinine, xylose clearances) will be of no more value than determination of urea clearance. The normal urea clearance in hypertension is not an expression of functional renal integrity, since it is usually associated with renal ischemia, but, on the contrary, is usually evidence of renal vasoconstriction, which may be intense. For example, an increase of filtration fraction from 0.20, a normal level, to 0.30, a value not infrequent in essential hypertension, is enough to maintain urea clearance at 100 per cent of normal at a time when renal blood flow has fallen from a normal level of 1,000 to an ischemic level of 600 c.c. per minute.

Technically, the measurement of the simultaneous clearances of diodrast and inulin is not difficult, although it is necessarily more painstaking than routine determination of urea clearances. It is our view that the advantages of these determinations outweigh the difficulties of technique. Briefly, diodrast and inulin in sterile 0.9 per cent sodium chloride solution are given by slow intravenous infusion after the more rapid injection of a concentrated priming dose of the mixture. When it is believed that the plasma levels of diodrast and

inulin have reached a satisfactory level, which will be maintained by the infusion, the patient is catheterized, the bladder is rinsed out with sterile saline, and the urine and washings are rejected. A sample of blood is then obtained, and the bladder is again washed and urine is collected in about twenty minutes. Urine and washings are combined, and the time washing is completed is noted as the end of the first clearance period. Subsequent periods are collected in the same manner, although, with a constant infusion, sampling of blood is necessary only in every other period. We have adopted the routine collection of three urine specimens and two blood samples, and find this satisfactory unless unusual difficulty is noted in the collection of urine, in which case further collections are made. The necessity of intravenous infusion may be avoided by subcutaneous injection of a concentrated solution of diodrast, inulin, and novocain,[43] and the subsequent determination of clearance is made during a fall in plasma concentrations of diodrast and inulin, so that blood samples should be obtained in each period. Clearance is calculated as UV/P, where U equals urine concentration in milligrams per 100 c.c. and V is the volume of mixed urine and washings in cubic centimeters per minute, while P is the mean plasma concentration during the clearance period expressed in milligrams per 100 c.c. The value of P is obtained by interpolation from observed values to a point in each period approximately one minute before the midpoint of each period, thus allowing a delay time of one minute for the passage of urine down the renal tubules and ureters. Methods of diodrast analysis have been described by Smith, Goldring, and Chasis,[44] and by White and Rolf.[45] Methods of inulin analysis in present use are those of Alving, Rubin, and Miller,[46] and of Corcoran and Page.[47] We are at present using a slight modification of the method of White and Rolf for diodrast analysis.

The simultaneous measurement of diodrast and inulin clearance may be extended to the measurement of the maximum capacity of the kidney to secrete diodrast. Whereas efforts are made in the determination of renal plasma flow to maintain renal diodrast load (renal plasma flow × plasma diodrast concentration) at levels such that the capacity of the kidney to transfer diodrast is not taxed and the removal of diodrast from blood is thus kept at a maximum, the determination of maximum capacity to secrete diodrast demands that the diodrast load be sufficient, so that secretory capacity of the kidney is overtaxed. Under such conditions, the diodrast excreted by the kidney by secretion (diodrast present in urine—diodrast excreted by filtration) reaches a constant level, which is expressed in milligrams per minute. The value expresses the maximum capacity of the diodrast transfer mechanism and, since the mechanism of transfer depends upon activity of the tubule cells, it follows that the maximum diodrast secretion rate is an expression of the mass of functioning tubular tissue. This value has been denominated Tm_D (tubular mass, diodrast). Clearly, interpretation of the absolute levels of diodrast and inulin clearances with references to Tm_D is a more accurate expression of the status of renal hemodynamics than is their reference to average normal values, since thus stated, the value $\frac{\text{diodrast clearance}}{Tm_D}$ is an expression of cubic centimeters of plasma flow per unit of tubular tissue. Errors of interpretation due to differences in kidney size

in people of similar surface area are, therefore, excluded. Hypertensives, whose diodrast clearance may be normal with reference to surface area, may by this means be shown to have a decreased blood flow in cubic centimeters per unit of tubular tissue,[42] i.e., with reference to renal mass.

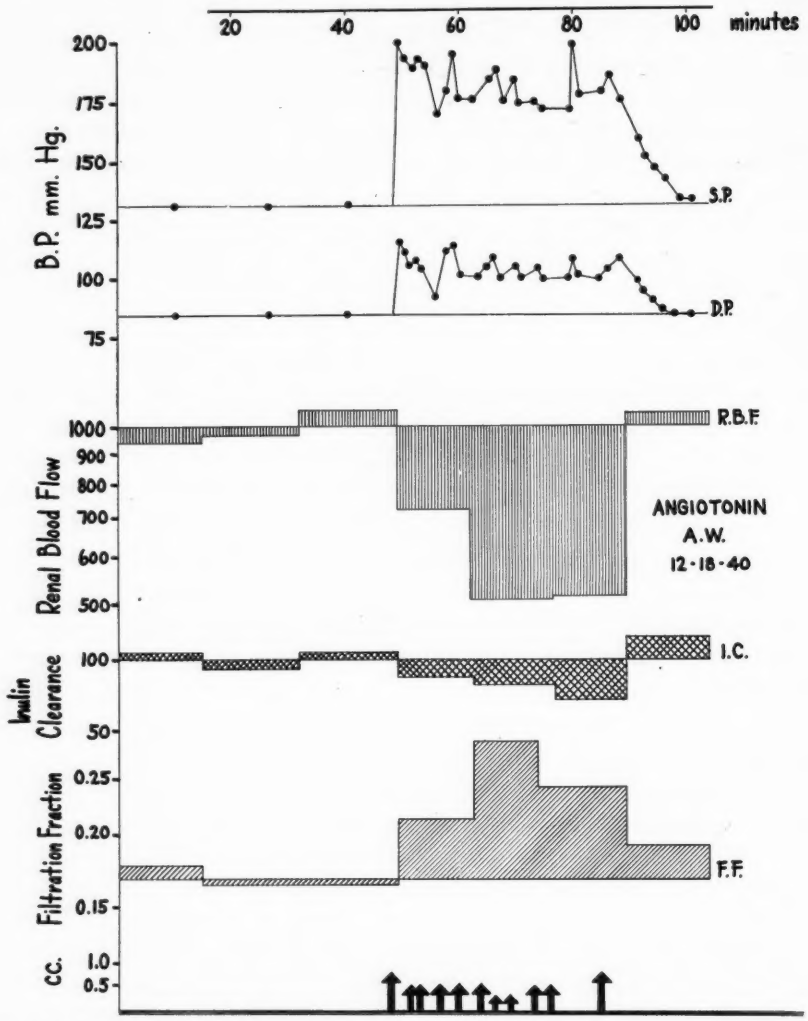

Chart 3.—Effects of infusion of angiotonin on arterial blood pressure, renal blood flow, inulin clearance, and filtration fraction in patient A. W. Ordinates from above downward: arterial systolic (S.P.) and diastolic (D.P.) blood pressure; effective renal blood flow (R.B.F.) in cubic centimeters per minute; inulin clearance (I.C.) in cubic centimeters per minute; filtration fraction (inulin/diodrast clearance ratio, F.F.); cubic centimeter indicates volumes of initial intravenous injection of angiotonin and of subsequent injections into the infusing fluid. Effective renal blood flow, inulin clearance, and filtration fraction are plotted semilogarithmically. Abscissa: time in minutes.

Application of these methods to the study of renal function is shown in Charts 3 and 4. Chart 3, which illustrates the effect of angiotonin on renal circulation, also demonstrates the similarity of the functional change due to angiotonin to that which is present in hypertension. Injection of angiotonin in human beings increases arterial pressure and greatly decreases renal blood flow, but decreases only slightly the rate of glomerular filtration, because, as in hyper-

tension, there occurs increased extraction of water from the glomerular plasma (increased filtration fraction).[48] Angiotonin, therefore, constricts the glomerular arterioles. Its renal action is such that it may be the pressor substance of hypertension. In this connection, it should be noted that the renal ischemia

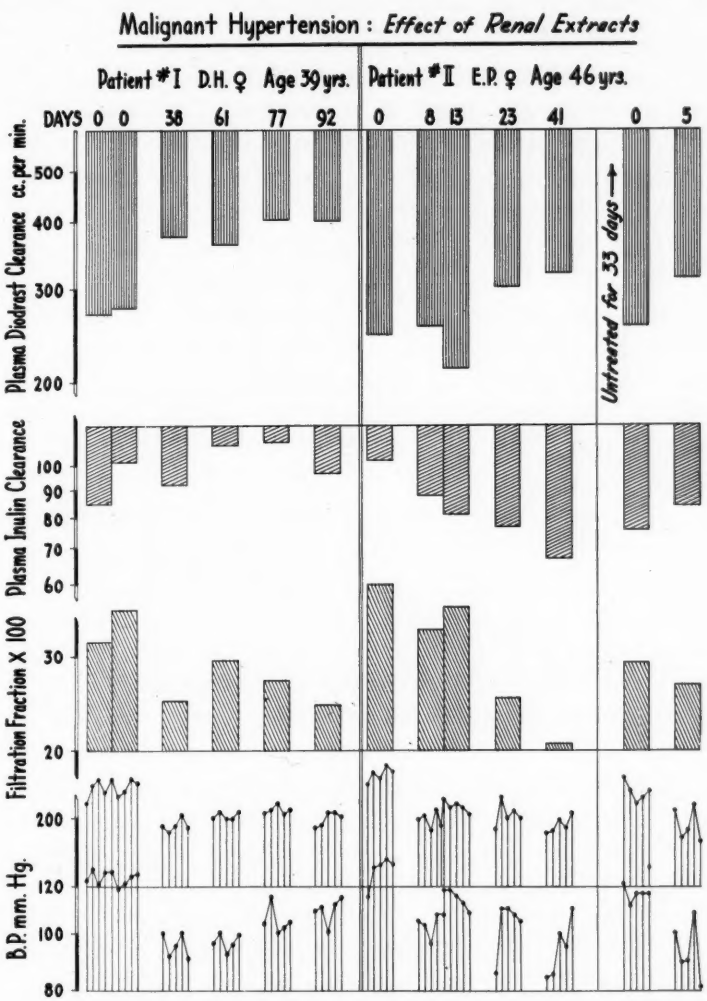

Chart 4.—Action of renal extracts on renal hemodynamics and arterial pressure in malignant hypertension. Numerals on abscissa refer to days in each series of intramuscular injections of renal extracts, while ordinates are referred to average normal values.

of hypertension, if due to angiotonin, is more probably a result of the action of the vasopressor system than a cause of the condition. A similar, apparently secondary, renal hemodynamic abnormality is present in the diastolic hypertension associated with coarctation of the aorta.[49] A further similarity of angiotonin to the possible pressor agent of hypertension, and one which excludes adrenin and pituitrin, is the fact that angiotonin exerts its pressor action without causing pallor or a decrease of skin temperature.[50] This is also true of renin.[41, 51] Others have shown that the acute pressor action of angiotonin is associated with greatly increased peripheral resistance and with decreased

cardiac output[52] and increased venous pressure,[53] the latter possibly the result of the heart's emptying against great resistance. Angiotonin also increases the force of the heartbeat[54] and cardiac work and efficiency, but it decreases diastolic cardiac volume.[55] It is interesting to speculate as to whether or not angiotonin would decrease cardiac output in the case of the hypertrophied heart of a long-standing hypertensive person.

Studies of normal renal hemodynamics by the clearances of diodrast and inulin have shown an apparent autonomy of renal circulation, which is largely exercised by variations in caliber of the efferent arterioles.[56] The normal tendency of the autonomous renal circulation is to respond to variations of systemic blood pressure and supply by changes of efferent arteriolar tone which maintain filtration rate near its normal level, at the same time varying the rate of renal blood flow. Since, as has been noted, renin may be liberated in response to pressure changes in the afferent arteriole, it is not illogical to assume that the renal vasopressor system may have a normal function in the autonomy of renal circulation, and hypertension may be an abnormal release of this mechanism. Teleologically, it is difficult to imagine a function of this system in the maintenance of normal arterial pressure, since the normal blood pressure level is not affected by bilateral nephrectomy.

Diodrast and inulin clearances have also been used in the study of the renal changes that result from the treatment of patients suffering from hypertension with renal extracts containing the renal antipressor, or with other agents. The decreased arterial pressure following sympathectomy in hypertension,[57, 58] or the therapeutic use of thiocyanate,[59] is not usually associated with the undoing of the efferent arteriolar constriction, and it may be assumed from this that neither sympathectomy nor thiocyanate reverses the process that leads to increased arterial pressure and renal vasoconstriction.

The action of renal antipressor extracts, on the contrary, is associated with a concurrent decrease of arterial pressure and evidence of efferent arteriolar relaxation, an observation that adds to the evidence which suggests that these extracts in fact inhibit the action of angiotonin and the renal vasopressor system in hypertension[60] (Chart 4). The extent to which renal blood flow is affected by renal vasodilatation depends upon the balance between renal vasoconstriction, vasosclerosis, and arterial pressure, so that it is not greatly increased when renal vasodilatation is associated with decreased arterial pressure.

The measurement of diodrast, phenol red, and inulin clearances has also yielded interesting information as to the renal functional changes present in pre-eclamptic and eclamptic toxemia of pregnancy.[61] The succession of these changes in two patients with eclampsia before and after delivery is shown in Chart 5. Briefly, pre-eclampsia and eclampsia were characterized by a decrease of filtration fraction, which seems to be an expression of swelling of the glomerular capillaries, since it is not usually associated with decreased renal blood flow. Other patients suffering from "toxemia of pregnancy" show an increased filtration fraction and a decreased renal blood flow. It is believed that in these latter the renal changes are an expression of a process identical with that of essential hypertension.

It will be noted that the afferent arteriole has been largely neglected in the foregoing discussion, in which emphasis is placed on constriction of efferent arteriole in hypertension. Structural, as opposed to functional, studies of the kidney in early hypertension have shown that the characteristic lesion is not situated in the efferent arteriole, but that it consists of hyalinization and sclerosis

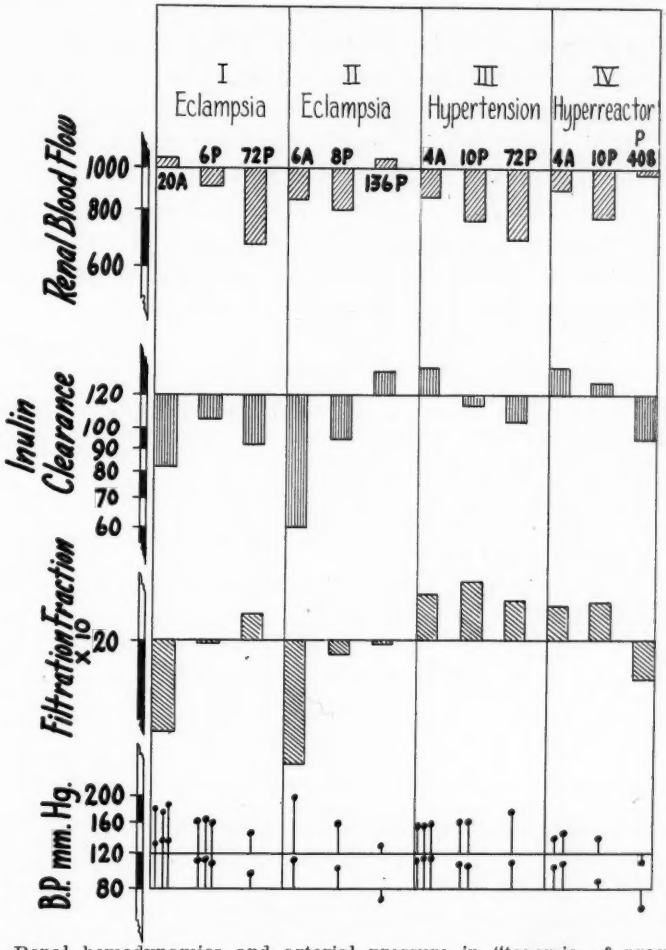

Chart 5.—Renal hemodynamics and arterial pressure in "toxemia of pregnancy." The numerals on abscissa refer to the day of observation in number of days ante (*A*) or postpartum (*P*). These cases were referred to elsewhere[61] as patients No. A1, A2, B4, and B3.

of the afferent arteriole. Afferent arteriolar sclerosis may, in fact, be widespread at a time when there is little evidence of abnormal arteriolosclerosis elsewhere in the body.[62] A possible explanation of this curious opposition of structural and functional changes may be obtained from consideration of vasosclerosis as it occurs in experimental renal hypertension. In this condition, the kidney is exposed, as are other organs, to the vasoconstrictor, apparently angiotonin or a similar substance, which is present in systemic blood, while it is at the same time protected from the increase of arterial pressure by the restriction of a clamp on the renal artery or a scar around the parenchyma. The kidneys of such animals rarely show evidences of sclerosis or of arteriolonecrosis in malignant hypertension, although these changes are widespread in other organs[5] and

may be present in the contralateral "normal kidney."[63] The vascular degenerative changes are, therefore, apparently due in part to the increase of systemic arterial pressure and not alone to the presence of vasoconstriction. It is, therefore, altogether fitting that sclerosis should occur in the afferent (high pressure) arteriole at a time when it is absent in the efferent (low pressure) arteriole of the kidney.

The early afferent arteriolar sclerosis of hypertension has at the same time a significance other than that of a simple result of the action of increased arterial pressure, for, if it were present from other causes *before* the onset of hypertension, it would act effectively as would "myriads of little clamps."[5] Evidence of such a change prior to the onset of hypertension is unavailable, although the wide distribution of renal sclerosis in early hypertension is at least suggestive.[62]

The final origin of hypertension remains obscure, for the demonstration of the part played by the renal pressor system clarifies some aspects of its etiology but, at the same time, poses new problems.

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Addendum: Recently published observations correlate the diastolic arterial pressure of patients with hypertension with the rates of renal blood flow and glomerular filtration (Friedman, M., Selzer, A., Rosenblum, H.: The Renal Blood Flow in Hypertension, J. A. M. A. **117**: 92, 1941). A new method for the determination of diodrast has also been described (Alpert, L. K.: A Rapid Method for the Determination of Diodrast-Iodine in Blood and Urine, Bull. Johns Hopkins Hosp. **68**: 522, 1941).

CLINICAL AND EXPERIMENTAL

CAPILLARY STUDIES IN RAYNAUD'S DISEASE*

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INTRODUCTION

EXACT capillaroscopic investigations and functional tests in cases of Raynaud's disease in general, as well as in those studied before and after complete elimination of sympathetic innervation, promise to yield significant data as to the role of the autonomic influences on the capillary. The relationship to the neuroses which have found their symptomatic expression in this part of the circulatory system may also be elucidated.

This study is an investigation of the constitutional capillary disturbances, the functional processes in the capillaries and the capillary permeability in cases of Raynaud's disease. These factors were studied both before and after preganglionic sympathectomy.

It is impossible to discuss the work of the many outstanding investigators who have been interested in these problems from the physiologic and psychologic viewpoints.[1-6, 11, 13-16, 19, 22, 23, 31, 33, 36] Raynaud himself suggested that emotional stress can precipitate an angiospastic attack.[29] In recent years the most extensive survey of the various concepts of Raynaud phenomena has been published by Hunt[17] who states that in 50 per cent of the cases of Raynaud's disease the attacks are precipitated by emotional factors (surprise, fatigue, etc.). He denies, however, any relationship between this illness and the psychoneuroses. That psychic stimuli may cause attacks is a point also stressed by Simpson, Brown, and Adson,[30] and by White.[35] Recently, Mittelmann and Wolff[25] discussed the interplay between affective and physical environmental influences in a case of Raynaud's disease in which they could show the significance of emotional factors in the precipitation of the attacks.

Mueller and Parisius[27] were among the first to call attention to the relations of capillary disorders to the neuroses. They found "imbalance, disharmony, and dysergia in the psychic as well as in the organic sphere" to be characteristic for the vasoneuroses in general. These features are manifested in the form and function of the capillaries. According to these authors, a very frequent characteristic of the vasoneuroses is the "spastic-atonic symptom complex," "vasoneurotic diathesis," that is, not only prolonged widening and narrowing of the capillary lumina, but also, either simultaneously or in quick alternation, dilata-

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tion and constriction. In all the more severe vasoneuroses the flow is extremely changeable and irregular. At times the stream flows quietly and evenly, then very quickly; at times it is sluggish and "granular," then it ceases completely for a brief interval. Often these various phenomena can be seen simultaneously in different capillary loops in the same microscopic field. Often a continuous capillary flow becomes granular. The "granular flow," in addition, may begin in the arterial as well as the venous limb. At times even spontaneous refluxes from the venous to the arterial limb are found, a phenomenon previously thought to be seen only in marked congestive failure.

In the majority of vasoneurotics Mueller and Parisius found an increased number of somewhat congested papillary capillaries and more vessels in the subpapillary plexus. (They examined the skin of the interclavicular groove.) In a small number of cases no abnormal overfilling of the vessels was seen; on the other hand, often no capillaries at all were visible. The size of the capillaries also deviates from the norm. Frequently there are long and thin loops, at times very short, thick, irregularly distributed loops. The most striking, and indeed the most pathognomonic, capillary form which is found in vasoneurotics is the irregular rete mirabile. At the nailfold the loops are not quite parallel; rather, they converge or diverge in an indefinite fashion. As a rule, the arterial limb is very narrow, the venous rather wide and irregular; a variable number of anastomoses connect the individual loops. Finally, the subpapillary plexus is usually visible and shows very wide vessels. Besides the characteristics mentioned above, according to Mueller and Parisius, there is also a functional disturbance in the permeability of the capillary walls which can be seen microscopically in the more severe forms of the vasoneuroses, is the hemorrhagic diapedesis, occurring either spontaneously or after slight mechanical trauma. The above capillary picture, as defined by Mueller and Parisius, was later confirmed by many other authors.

MATERIAL

From the capillaroscopic investigations of a series of cases presenting pathologic conditions, 42 patients, 29 of whom had Raynaud's disease, were selected for the purposes of this study. The other 13 patients were included either for purposes of comparison because of the similarity of the symptomatic picture, as, for example, Buerger's disease, or because, for one reason or another, a sympathectomy was performed and they, therefore, seemed to be of particular interest.

Forty-two hands of nonoperated patients were examined. Forty-six hands were of patients who had had a sympathectomy at the time of examination; 16 of these had also been seen before operation. Frequently the same patient was examined several times, particularly if an operation had been performed, in order to be able to observe the capillary changes at various times postoperatively.

METHOD

Before the capillaroscopic examination was performed, the following clinical data were obtained: The body habitus and its deviation from the standard types was noted. The color, pigmentation, approximate temperature, degree of moisture or dryness, fat content and separability of the skin from the subcutaneous

tissue were indicated. The skin was tested for dermographism. The vasomotor instability was checked by the absolute pulse rate and by the respiratory arrhythmia during inspiration and inspiratory apnea (Valsalva test). The systolic and diastolic pressures and their response to pressure on the carotid sinus were recorded.

CAPILLAROSCOPY

A Zeiss capillaroscope with a 60× objective and a 10× eyepiece was used. Since the small lamp within the tube does not offer adequate elimination, a special lighting apparatus for this purpose was attached. A special attachment permitting simultaneous observation and photography was inserted.

The morphology of the capillaries was described, with particular reference to developmental disturbance (archicapillaries, subpapillary plexus).[18] The corium was described as being well scalloped, not well scalloped or straight.

The number of capillaries per field in the first row was counted.

The length and width of the capillaries and changes in their appearance were noted.

The flow in the capillaries was described as granular or uniform, slow or fast, intermittent or continuous, and the appearance of "gaps" and periods of stasis were noted.

In addition, the following tests were done in order to gain better insight into the functional responses: The flow in the capillaries was studied after occluding the arterial and venous flow by the use of a blood pressure cuff quickly pumped up to 30 mm. above systolic blood pressure. The "reflux time,"[34] the eventual reversal in flow from the venous side to the arterial and any other changes appearing in this experiment were noted, with particular attention to the widening, narrowing, or disappearance of capillaries.

Finally, by slowly reducing the pressure in the sphygmomanometer cuff (about 10 mm. per 2 seconds), the height of the pressure at which the capillary flow reappeared was determined. This value we termed "critical capillary pressure."

A phenomenon to which special attention was paid was the occasional appearance of a light-refracting, collagenous tissue fluid which exuded from the large capillaries and obscured them. This was seen spontaneously as well as in the tests for reflux time. As will be explained later, this may be regarded as direct expression of increased capillary permeability.

With a special photographic apparatus, a series of colored films was taken which recorded the capillaries themselves in addition to the shading of the surrounding tissue. However, more distinct pictures were obtained by placing a green filter in front of the light, and the black and white photograph with panchromatic Agfa or Kodak film combined the advantage of a clear, sharp picture, with facility in making any desired enlargement.

When it was of no particular importance to record the width of the capillaries, but of more importance to obtain the general outline, a slight congestion of the capillaries, produced by the blood pressure cuff (50 to 60 mm.) was shown to be most advantageous in obtaining clear pictures. Of course, such photographs, taken in a congested state, cannot be used for the determination of the

actual width of the capillaries; on the other hand, if anastomoses to the vessels of the subpapillary plexus are visible microscopically, these become much clearer and are then more easily photographed.

An attempt on the part of different observers to draw the picture the same size as seen in the microscope will give varied results and will lead to pictures of very different dimensions. Keeping in mind Krogh's criticism[19] of the existing capillary pictures in the literature and the lack of data on the degree of magnification, we used the following method in our study: We set up a counting chamber for red blood cells under the microscope and photographed it; thus we had a sharply delineated scale for all sharply focused capillaries. Our original photographs were thus magnified 18×.

By means of the picture of the counting chamber it was not difficult to prepare enlargements giving a definite magnification of the actual size of the object. We merely had to place a scale on the projection surface of the enlargement apparatus and arrange it in such a way that, for example, the intermediate space of the counting chamber which is $\frac{1}{20}$ mm. long, appeared exactly 2 cm. long, in order to obtain a 400× enlargement.

By this method errors in the determination of the size of the microscopic picture can be avoided. Brown[5] made such errors; he later had to correct his measurements because of incorrect calculation of the scale. Similarly, the photographs of Duryee and Wright[11] are incorrectly computed. In their paper two photographs are reproduced with the statement that the enlargement is "176 times on the original film" and "the enlargements shown are about four times the size of the original film." That means then that the picture reproduced was enlarged 176 × 4 or 704×. In order to check their data we measured the largest capillary loop in their picture; it was 5 cm. long. In reality it would then be 50/704, or 0.071 mm. long. This would be only 10× as large as the average red blood cell. There must, therefore, be a mistake in their calculation.

REFLUX TIME AND CRITICAL CAPILLARY PRESSURE

The method used is to encircle the upper arm with a blood pressure cuff which is quickly pumped up to 30 mm. above systolic blood pressure. The time required for the blood in the capillary under observation to stop flowing is the "reflux time," which under normal conditions averages fifteen seconds. Lange[21] found a lower value in normal persons. We must go well above systolic pressure to be sure the arterial flow is completely shut off. It is important to wait several minutes before the test is repeated, because this length of time is necessary before the venous congestion completely disappears.

When the compression is released, the capillary flow reappears in normal persons at a pressure between 10 and 25 mm. below the systolic blood pressure. This we have termed the "critical capillary pressure." A reappearance above or below these limits must be considered pathologic.

NORMAL CAPILLARY PICTURES IN ADULTS

By means of capillaroscopy one sees the following picture at the nailfold of normal adults: The boundary between the cutis and corium is well scalloped. The capillaries of the first row (limbus capillaries) are, for the most part, hair-

pin-like; some show loops or figure-of-eight forms. They are usually parallel. The arterial limb is narrower than the venous and allows blood corpuscles to pass through only one by one. According to Mueller,[20] the patent width of the lumen is 0.009 to 0.012 mm. The venous limb is wider and allows two or more blood cells in a row to pass through. The width of the lumen, according to Krogh,[19] is 0.02 mm.

The height of the capillaries, that is, the straight line between the foot of the capillary, where it is just visible, to the convexity of the arteriovenous transitional loop is about 0.2 to 0.4 mm. The distance of the capillaries from one another is approximately uniform. The number of capillaries in the first row is about 7 to 10 per millimeter. The distance of the heads of the capillaries from the corium is about 0.1 to 0.3 mm. The connection between these capillaries and deeper vessels is normally not visible.

Behind the above-described row of capillaries, which is just adjacent to the corium, there is usually a second, and often a third, row of shorter, but otherwise similarly formed capillaries. In the deeper layers no arrangement in rows can be made out, but one sees rather chessboard-like patterns or scattered, short loops which represent the capillary heads.

Normally the subpapillary plexus is invisible.

The above-mentioned loops and figure-of-eight forms, if present in the minority, are considered normal; so, too, are slight deviations from the very regular structure, such as slight variations in the length of the capillaries or a slightly unequal spacing.

The flow in normal capillaries is usually smooth and even and of moderate speed, but a "granular" type of stream is sometimes seen and occasionally isolated capillaries seem to disappear. Actually this is due to the fact that they contain no blood corpuscles, since it is only these that are visible and not the capillary itself. At other times the circulation in isolated capillaries ceases entirely for a brief period of time. Both of these phenomena are to be considered normal.

CAPILLARY PICTURE IN RAYNAUD'S DISEASE

The appearance and behavior of the capillaries in Raynaud's disease depend on the severity of the disease and are different in the different color stages of the extremities. In order to have comparable observations one must always perform the examinations in a warm room at a standard temperature. In this warm room the capillaries observed at the nailfold of a patient showed the same picture, even on different days, as long as no operation was performed.

The typical picture in a severe case of Raynaud's disease (in a warm room) is the following: Most of the capillaries are enlarged. The presence of giant capillaries with a width of 5 to 8 red blood cell diameters or more is striking. The space between the two limbs of such a giant capillary is about 10 red blood cell diameters. Its shape may be ringlike or hairpin-like or irregular and tortuous. The flow in the capillaries is slow and sluggish, and often prolonged periods of stasis are observed. In some cases blood extravasates and thromboses are found. Very often there are very narrow, thin capillaries between the enlarged, giant ones.

In severe cases which are combined with sclerodermic changes, all the above-mentioned findings may be present, and in addition, the number of capillaries is smaller. The picture is very often hazy and the flow is barely visible; the stream, if visible, is very sluggish. In the reflected light the tissue has a brighter color than in other cases, probably due to the increased collagenous fibers. In addition, the excretory ducts of the sweat glands are often visibly enlarged and dilated, and the escape of drops of sweat may be seen. Since we observed this phenomenon in several cases, it may be evidence that there is a hyperfunction of the sympathicus in Raynaud's disease. This confirms Raynaud's original concept[29] that one of the mechanisms of the disease is an abnormal vasomotor tone. Lewis' opinion[23] is that in true Raynaud's disease there is a "local fault" and that the vasomotor tone is normal. Bishop, Heinbecker, and O'Leary[2] believe in a local, inherent, trophic, etiologic factor. In some advanced cases a sensitization to humoral substances is present and is sufficient to produce an attack.

In early and mild cases of Raynaud's disease the picture is not so striking; giant capillaries, thromboses, and blood extravasates are absent. The typical picture in mild cases of Raynaud's disease or vasoneurosis is as follows: very tortuous, twisted and bent capillaries, usually with a spastic, narrow, arterial portion and a dilated, congested, venous portion. A congested subpapillary plexus is usually visible.

REFLUX TIME

In severe cases of Raynaud's disease the reflux time shows a marked deviation from the norm. We frequently find capillaries in which, upon compression of the arteries of the arm, there is an immediate stasis (the decision as to whether it happened *post hoc* or *propter hoc* is sometimes difficult, since spontaneous stases often occur). In the enlarged, giant capillaries, in which the flow before compression was very slow and sluggish, and in which there were frequent, spontaneous stases, we often observe that after compression this flow remains visible for a long time before it finally stops. Time intervals of more than one minute are not rare. (The experiment should not be repeated immediately; one must wait several minutes, otherwise the results will be incorrect.) As is to be seen from these contradictory findings under the seemingly identical conditions, we are concerned with a complicated function which apparently represents the resultants of various factors influencing the reflux time. This will be discussed later.

CRITICAL CAPILLARY PRESSURE

Observations of the reappearance of the capillary flow after compression show varying results in different cases. In several examinations of the same case at different times there is, for the most part, a rather constant pressure at which the flow reappears. The experiments must not be done immediately after one another; a rest of several minutes must be interposed. Those patients in whom the pressure at which the flow reappears varies on different examinations seem to belong to that group in which the blood pressure also changes frequently. In general, the capillary flow reappears normally at a pressure of about 10 to 25 mm. below the systolic blood pressure. A striking and abnormal finding was

Difference Between Systolic Pressure and Critical Capillary Pressure

Name	Clinical Classification	Systolic Blood Pressure	Critical Capillary Pressure
Che	C	115	100
McD	C	115	90
Rou.E	C	94	82
Bur	R_1	118	100
Cla	R_1	100	75
Goo	R_1	100	75
Mar	R_1	124	120
Pop	R_1	128	120
Sey	R_1	110	80
Sch	R_1	118	90
Sut	R_1	112	90
Syp	R_1	105	98
Wes	R_1	110	90
Bor	R_2	125	60
Buc	R_2	115	80
Lab	R_2	130	95
Lam	R_2	125	90
Lan	R_2	110	65
Lop	R_2	135	110
Wic	R_2	115	60
Dwy	R_3	120	60
Hor	R_3	112	80
Pic	R_3	105	55
Pro	R_3	118	60
Rou.M	R_3	115	90
Tom	R_3	110	40
Whi	R_3	164	60

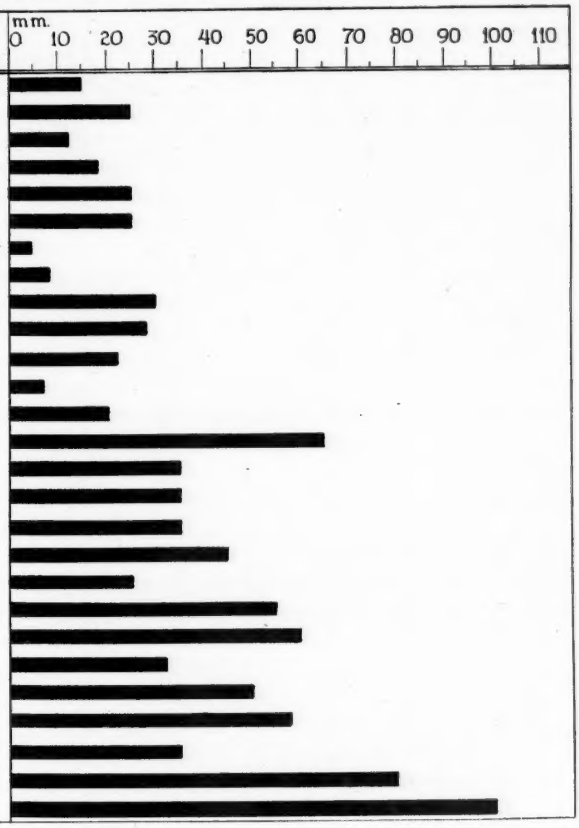


Chart 1.

Difference Between Systolic Pressure and Critical Capillary Pressure

O = before operation < = after operation

Name	Clinical classification
Che	C
Syp	R_1
Buc	R_2
Lab	R_2
Lam	R_2
Lan	R_2
Wic	R_2
Hor	R_3
Pic	R_3
Pro	R_3
Whi	R_3

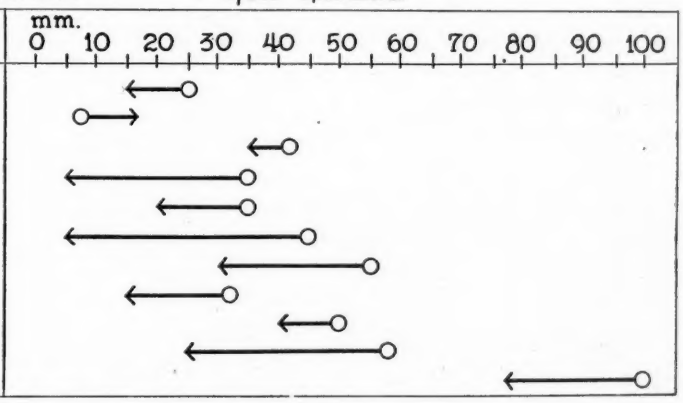


Chart 2.

TABLE I

CHIEF CAPILLARISCOPIC FINDINGS

NAME	CLINICAL CLASSIFICATION	OPERATION	SLUGGISH FLOW	GIANT CAPILLARIES	RESUMPTION OF FLOW 30 MM. OR MORE BELOW SYSTOLIC PRESSURE
Cla	C	R–	+	–	–
		L–	+	–	–
Fre	C	R–	+	–	·
		L–	–	–	·
Rou. E	C	R–	+	–	–
		L–	·	·	·
Bur	R_1	R–	–	–	–
		L–	·	·	·
Che	R_1	R–	–	–	–
		L–	·	·	·
Cron	R_1	R+	+	–	·
		L+	+	–	·
Goo	R_1	R+	–	–	–
		L+	+	–	+
McD	R_1	R+	–	–	–
		L–	–	–	–
Mar	R_1	R–	–	–	–
		L–	·	·	·
Pop	R_1	R–	+	–	–
		L–	·	·	·
Sey	R_1	R–	+	–	·
		L+	·	·	·
Sch	R_1	R+	–	–	·
		L+	·	·	·
Sut	R_1	R–	+	–	+
		L–	·	·	·
Syp	R_1	R–	+	+	–
		L–	·	·	·
Wes	R_1	R+	+	–	–
		L+	+	·	–
Bor	R_2	R+	+	+	+
		L+	·	·	·
Buc	R_2	R–	+	–	+
		L–	·	·	·
Lab	R_2	R–	+	–	+
		L–	·	·	·
Lam	R_2	R–	+	+	+
		L–	+	+	+
Leh	R_2	R–	+	+	·
		L–	·	·	·
Lop	R_2	R–	–	–	–
		L–	–	–	+
McG	R_2	R+	+	–	–
		L+	–	–	–
McI	R_2	R+	–	+	·
		L+	–	+	·
Sir	R_2	R+	·	+	·
		L+	·	·	·
Wic	R_2	R–	–	–	+
		L+	·	·	·
Dwy	R_3	R–	+	+	–
		L+	–	+	–
Gal	R_3	R+	–	+	·
		L–	·	·	·
Hor	R_3	R–	+	+	–
		L–	–	+	–

R = Raynaud's disease.
C = Causalgia.

TABLE I—CONT'D

NAME	CLINICAL CLASSIFICATION	OPERATION	SLUGGISH FLOW	GIANT CAPILLARIES	RESUMPTION OF FLOW 30 MM. OR MORE BELOW SYSTOLIC PRESSURE
Lan	R_3	R–	+	+	+
		L–	+	+	+
McC	R_3	R+	–	–	–
		L–	+	–	–
Pic	R_3	R–	+	+	+
		L–	.	.	.
Pro	R_3	R–	+	–	–
		L–	.	.	–
Rou. M	R_3	R+	+	+	–
		L+	+	–	–
Tom	R_3	R+	+	+	+
		L+	.	.	.
Whi	R_3	R–	+	+	+
		L–	.	.	.

obtained in severe cases of Raynaud's disease and sclerodermia in that the pressure often had to be reduced 60 mm. below the systolic pressure before the capillary flow reappeared. Reappearance of the flow at a point 35 mm. or more below the systolic pressure seems to be due to a local disturbance in capillary function.

In Charts 1 and 2 this relationship is graphically presented. In Chart 1 the difference between systolic and critical capillary pressure is shown in comparison with the severity of the illness; in Chart 2 the differences before and after operation may be seen.

CAPILLARY PERMEABILITY

As mentioned above, in severe cases one frequently may see blood extravasations. This is to be regarded as a sign of increased capillary permeability. Moreover, in a few cases with giant capillaries, we were able to observe the following phenomenon: After the blood pressure cuff was pumped up, a light-refracting substance gradually began to cover these giant capillaries, and the entire field became hazy. This may have been due to a filtration of plasma through the capillary wall because of increased capillary permeability. The criticism that this phenomenon may be explained by a difference in the refraction of light by the giant capillaries lying beneath can be refuted by the fact that we had the opportunity to observe the appearance of this "shiny substance" after prolonged compression in cases in which it was not formerly present. It is, therefore, due to the passage of plasma substance through the capillary wall. In a subsequent examination at another time the capillary picture was again distinct. This may be considered as further proof in favor of our interpretation.

Patients whose fingers were edematous usually showed a vague, hazy, capillary picture. More often it was seen that the originally clear capillary picture became vague and hazy upon prolonged compression, indicating increased permeability. Capillary permeability and its correlation to adrenergic and cholinergic reactions was investigated by the autonomic skin test.[10]

CLINICAL AND CAPILLARY GROUPING

In Table I the results of the capillaroscopic examination are compared with the classification of the cases from the clinical and surgical viewpoints. The characteristics which are significant and particularly suitable for a tabular survey are as follows:

1. The presence of giant capillaries (noted in the table by +).
2. Sluggishness of flow (noted in the table by +).
3. The reflux time and the critical capillary pressure (see Method). If the difference between the systolic blood pressure and the critical capillary pressure is above 30 mm., this is noted in the table by +; a – denotes that the difference is less, and a dot that the experiment was not done.

The clinical grouping, from the surgical viewpoint, is as follows:

1. Group 1 of Raynaud (R_1): no obvious local changes on ordinary examination of the affected extremity.
2. Group 2 of Raynaud (R_2): obvious local changes.
3. Group 3 of Raynaud (R_3): obvious local changes and x-ray abnormalities such as bone atrophies.
4. Causalgia (C): pain a prominent symptom with secondary vasomotor changes.

In Table I we see that the plus signs tend to form a group which includes those patients who were also designated as group R_2 or R_3 clinically, whereas the groupings R_1 (mild cases) and C (so-called cases of causalgia) were frequently characterized in the table by minus signs. Insofar as we examined these patients preoperatively, these findings are noted in the table by minus signs. Those patients who were seen only after operation are noted in the table by plus signs. It may be concluded from experience with cases seen before and after operation that the above cases may also have had a pathologic picture before operation, i.e., giant capillaries and sluggish flow.

A comparison of the capillaroscopic and clinical groupings of Raynaud cases revealed certain discrepancies. For example, the nailfolds of Lop and Wic (Table I) showed less marked findings capillaroscopically than we would expect on the bases of the clinical classification, whereas Dwy and Goo showed more severe capillaroscopic findings. The past history of Lop reveals, however, that the signs and symptoms of the disease were localized only in the feet, never in the hands which were always free. She was placed in group 2 only on the basis of the findings in her legs, not in her hands. The patient actually had had only a lumbar sympathectomy. The seeming inconsistency in the classification is thereby explained.

As mentioned above, the capillaroscopic findings in patient Wic were those of a benign case, whereas the clinical impression was that of a moderately severe case. This case is here described in greater detail because of its other interesting features.

CASE REPORTS

CASE 1.—The patient was a young woman, aged 26 years, who impressed the physicians by her persistent complaints of pain in the hands and occasional blanching of the fingers. This was the reason for placing her in group 2, although according to the capillaroscopic exam-

ination she belonged in group 1. Psychologically, she had the personal characteristics of an obsessional neurotic. She showed interesting changes in the capillary picture upon repeated examinations at different times.

She was examined for the first time four months after a left-sided preganglionic sympathectomy. The left hand was dry and warm, the right hand moist, cool, and purple. Only the right hand was examined capillaroscopically at that time. There were signs of a developmental disturbance of the capillaries (expressed in the persistence of the subpapillary plexus and in visible anastomoses with the papillary capillaries) and a flow of moderate speed, a condition usually not found in moderately severe cases of Raynaud's disease.

When the patient was examined eight months after operation on the left hand and four months after operation on the right hand, she was still under the influence of a recent gynergen injection. She said that she was in the habit of taking these injections originally because of asphyxia of the finger, but now because of migraine; she claimed that these injections helped her. Her hands were cold, dry, and showed purplish spots when she entered the room. The most striking capillaroscopic finding was a very marked sluggishness of flow; often there was a complete lack of motion in the microscopic field.

Without being asked, but quite spontaneously, the patient gave the following information: Before the sympathectomy, whenever she had a gynergen injection, the hands became reddish, warm, and moist; since the operation, after a gynergen injection, her hands always became cold and purplish.

One month later the patient was again examined; she had had no gynergen injections for several days. The hands were reddish pale; the palms were colored bright red and were dry and warm. Capillaroscopically there was a rapid blood flow in the capillaries. In contrast to the former findings, the stream never became sluggish and no periods of stasis appeared. The capillaries which, preoperatively as well as at the time of the first postoperative examination, without being under gynergen influence, were found to be dilated, now had a normal lumen. Thus these findings show that the gynergen injection before and after operation had a different effect on the appearance of the hands and of the capillaries.

The influence of gynergen on the asphyxia of the fingers before operation is comprehensible, because ergotamine paralyzes the sympathicus and, therefore, in cases of Raynaud's disease with spastic, constricted arterioles, there is dilatation of the arterioles and thereby a better blood supply to the hands. As a consequence of this the hands become warmer and redder. After sympathectomy the sympathetic action on the arterioles is absent, and the gynergen action has lost its pathway. Nevertheless, as the capillaroscopic examination reveals, it must still have an effect on the capillaries.

According to Cannon[6] and to our own findings, the sympathectomized zones are hypersensitive to adrenalin. Narrowing of the capillaries, which we found in most cases after sympathectomy, could be explained on this basis. There also seems to be an increased sensitivity to adrenalin-inhibiting substances, such as shown by the gynergen effect postoperatively. Capillaroscopic examination shows that after gynergen injection there is a dilatation of the capillaries in the sympathectomized region. Since this occurs without sympathetic regulation of the width of the arterioles, the consequence of the sudden, marked dilatation of the vessels is a slowing of the flow to the point of stasis. The cyanotic, blue-red color of the hands and the cool temperature are a result of this widening of the capillaries and the slowing of the flow.

The correctness of this assumption is confirmed by the third examination of the patient at a time when she had not received a gynergen injection. The findings were the same as in other sympathectomized patients: narrow capillaries, marked rapidity of flow, and warmth of the hands.

To summarize, the functional tests of the capillaries in the compression test in this patient may be discussed. Before operation on the right side (the left dorsal sympathectomy had been performed four months earlier) the reflux time was seventy-three seconds and the critical capillary pressure was 60 mm., that is,

55 mm. below systolic pressure (115 mm.). On examination of the right side four months after operation and while under the influence of ergotamine tartrate (gynergen), the reflux time in the right hand was thirty-five seconds and the critical capillary pressure was 80 mm., that is, 20 mm. below the systolic pressure of 100 mm. In the left arm, on which an operation was performed eight months previously, the reflux time was forty seconds and the critical capillary pressure was 84 mm., or 16 mm. below the systolic pressure.

One month later, without the influence of gynergen, the reflux time was twenty-five seconds on the right side and the critical capillary pressure was 90 mm., or 28 mm. below the systolic pressure (118 mm.).

Thus we see that the pathologic increase of the reflux time present before operation is significant, that postoperatively, with the patient under the influence of gynergen, it is less noticeable, and that without gynergen the reflux time is almost normal.

The variations in the critical pressure correspond to those in the reflux time. The pathologically low critical capillary pressure becomes distinctly higher after operation and, postoperatively, under the influence of gynergen, it is not so high as without gynergen. But since the blood pressure after the gynergen injection was only 100 mm., the difference between the systolic pressure and the critical capillary pressure (100 – 80 = 20 mm.) seemed somewhat less after the gynergen injection than later without gynergen (118 – 90 = 28 mm.).

CASE 2.—In the case of Goo there is also a discrepancy between the clinical picture and the capillaroscopic findings in the left hand. Clinically she was placed in group R_1, whereas the capillaroscopic findings were those of a more severe case.

She was a 33-year-old white, single woman, of slender build. Her skin was pale, her hands were cool and pinkish. On October 6, 1939, the date of examination, her pulse rate was 78 without any respiratory arrhythmia. Her blood pressure was 108/72.

Capillaroscopic examination of the right hand revealed a straight corium. There were 8 capillaries per field in the first row, irregular in form, most of them hairpin-like, but short, and some were half figure-of-eight shaped and widened. The width of the vessels changed during examination, some disappearing entirely. There was no marked difference between the arterial and venous limbs of the capillaries. The stream is slow and granular, and in some capillaries is invisible.

The second row reveals some hairpin-like, short, very widened capillaries. Others are dendritic and pointed. The subpapillary plexus is not visible.

Functional test: The blood stream stops after twenty-eight seconds, at a pressure of 130 mm. Hg., and starts again at 70 to 80 mm. Hg.

Capillaroscopic examination of the left hand reveals a straight corium. In the first row there are 6 widened, parallel capillaries, some hairpin-like, some twisted and dendritic. The venous limb is slightly wider than the arterial. The stream is sluggish and granular. In the second row the capillaries are diffusely located, some running horizontally. Some are very widened, sacklike shaped; some are short, some have biscuit forms. The subpapillary plexus is not visible; no archicapillaries can be seen.

Functional test: The stream stops at a pressure of 130 mm. after eight seconds and starts again at 65 mm. In a second test it stops after thirty-five seconds and starts again at a pressure of 55 mm. No retrograde flow is seen during compression.

Summary.—Irregularities in the form and location of the vessels. There are no developmental abnormalities of the capillaries, though there are obviously pathologic processes which lead to regenerative and degenerative changes. There are striking changes of the speed of the blood stream.

The subjective complaints of this patient contrasted strikingly with the objective findings in her hands, although she constantly reiterated that she had pain in her hands and legs. She was a severe obsessional neurotic whose interests were centered on sensations in her hands and legs. She insisted on procedures which gave her only transient relief. This explains the great number of operations which she underwent. They were as follows: at the age of 17, appendectomy; at 20, right ovarian cyst removed; at 31, left lumbar sympathectomy; at 32, in February, 1937, right lumbar sympathectomy; November 5, 1937, left dorsal preganglionic sympathectomy; November 13, 1937, right dorsal sympathectomy; December 16, 1937, left splanchnic resection; December 28, 1937, right splanchnic resection. On October 15, 1938, the patient had a right dorsal sympathectomy, and on November 14, 1938, resection of the fifth to the ninth left intercostal nerves. In spite of all these operations her neurotic complaints continued.

CASE 3.—Another case in which the clinical picture and capillary findings are not in agreement is the patient Dwy. The following are the salient features in this case:

Two and one-half years ago, in October, 1934, the patient crushed his left forefinger tip between two stones. He was seen immediately by his physician and after an x-ray had been taken, was treated conservatively at the local hospital. Later he was x-rayed again by an insurance company. The finger tip had become so sensitive, however, that he was unable to work because of his fear to touch anything with this hand. Two and one-quarter years ago the finger tip was amputated, but the extreme sensitivity remained. Whenever he touched anything, excruciating pain shot up the forearm. His sensitivity contrasted with the objective findings. Two years ago the pain sensation led to the diagnosis of neuroma, and the finger was amputated at the distal interphalangeal joint. No relief was obtained. One and one-half years ago another operation was performed, this time to remove the nerves on either side of the finger. The symptoms remained unchanged in spite of physiotherapy (massage, heat), and the finger seemed to be withering. Nine months ago another amputation was done, this time at the proximal interphalangeal joint, with no change in symptoms. A general physical examination at this time threw no light on the problem, and six "glass boot" treatments produced no change in the symptoms.

The patient has never before noticed numbness or tingling in the extremities or unusual sensitivity to cold. The sensitivity to touch and, more recently, extreme sensitivity to cold is now present in the whole left hand. On exposure to cold air the left hand turns numb and blue and requires ten to fifteen minutes to "thaw out."

On June 16, 1937, a left-sided sympathectomy was performed, with subsequent subjective improvement. The patient was examined for the first time eleven months later. Capillaroscopy at that time revealed in both hands a picture characteristic of a severe case of Raynaud's disease. On re-examination one year later (June 26, 1939) the changes were still more marked in the nonoperated hand. Even at the time of the first examination a large number of giant capillaries with a sluggish flow were found. Other capillaries showed a constantly changing rate of flow in that at times it was rapid and at times sluggish; occasionally it even stopped entirely for prolonged intervals. In addition, complete disappearance and reappearance of the capillaries was often observed. One year ago, in the right, nonoperated arm, the critical capillary pressure was 90 mm., that is, 30 mm. below the systolic blood pressure which was 120 mm. at that time, as it is now. In a follow-up examination on June, 1939, the critical capillary pressure was 60 mm.

From these findings we are justified in concluding that this patient must have had an asymptomatic Raynaud's disease at the time of his original injury. Afterward the accident precipitated a neurosis which revealed itself in his severe sensitivity and in the complicated postoperative course.

These examples show that severe capillary changes may be present in cases without or with inadequate clinical findings. In some cases neurotic complaints are in the foreground and antedate the visible clinical symptoms, since the basic capillary changes are not discovered.

CAPILLAROSCOPY BEFORE AND AFTER SYMPATHECTOMY

Before conclusions concerning the effect of the sympathicus on the capillary flow in Raynaud's disease are drawn from the comparison of capillaroscopic findings before and after preganglionic sympathectomy, the operation, as performed by Smithwick,[31] is described: "A vertical paravertebral incision centered over the third rib is made. Inner inch of third rib is resected. Second and third intercostal nerves are identified. Inner inch is resected, dividing both anterior and posterior roots, as well as the rami to the sympathetic trunk. The sympathetic nerve is identified and resected below the third ganglion, and the upper end of the section fold is sutured to the insertion of the muscles of the back. The incision is closed with fine silk."

The clinical results of the preganglionic dorsal sympathectomy are as follows: Whereas, before the operation, the affected hand is usually purple and mottled, cold and moist, after the operation it is pale, warm, and dry. It is worthwhile commenting that to the unbiased observer the hand on the sympathectomized side becomes not redder, but paler; and, as mentioned, it is much warmer than before the operation. The difference can be seen most clearly in those patients who have been operated on one side only.

The comparison of the capillaries at the nailfold before and after operation shows changes in the following: (1) the width of the capillaries, (2) the speed of the capillary blood stream, (3) thromboses and blood extravasates, (4) the reflux time, (5) the critical capillary pressure, and (6) the number of visible capillaries.

After sympathectomy in cases of Raynaud's disease a distinct *change in width* of the capillaries could be observed. In the mild cases the abnormal width of the capillaries was decreased. Of the two most severe cases with giant capillaries, in one the capillaries narrowed conspicuously, in the other they did not change; of 6 cases with widened capillaries, 5 showed narrowing after operation (Chart 3). Of the whole group of 15 operated cases, 9 showed narrowing after operation.

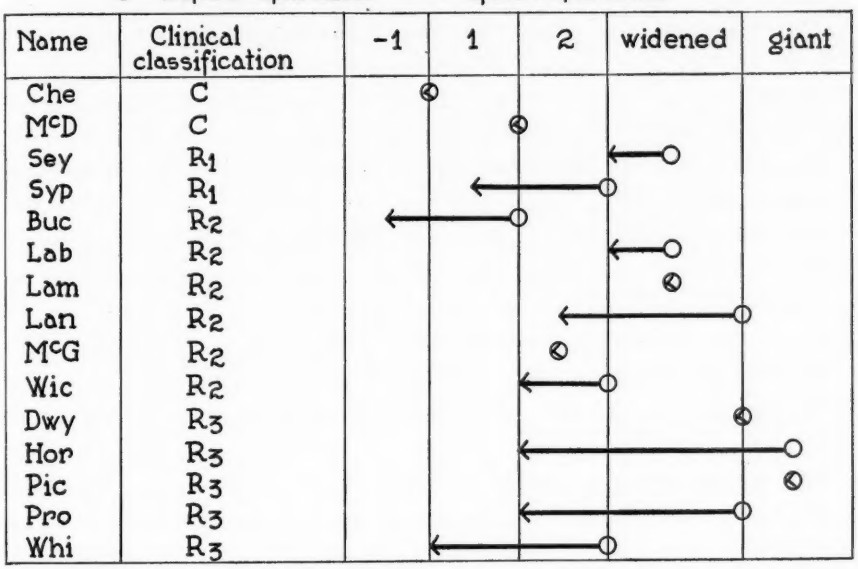
The most striking change after operation is, however, the increased *speed of the blood flow in the capillaries.* Of the 11 cases examined for speed of flow before and after operation, 2 showed no change and 9 showed a more rapid flow (Chart 4). In 3 others, examined only after operation, but where comparison could be made with the unoperated hand, all 3 operated cases showed increased flow. The changes are best seen in the mild and moderately severe cases. In the most advanced cases also, even after operation, a sluggish flow is still present in the markedly dilated capillaries, but one sees clearly the tendency to increased speed of the blood flow in the relative rarity and brevity of the periods of stasis as compared to the behavior before operation.

In two cases (Lam, Syp) on re-examination a few weeks after operation, *thromboses and hemorrhagic exudates* could no longer be found, although these were seen before operation.

In one case (Pic) where the increased capillary permeability before operation was characterized by the light-refracting substance in the neighborhood of the giant capillaries, such a "shiny" substance was not present after operation

Average Width of Capillaries *

O = before operation < = after operation



*The numbers -1, 1, etc., signify values of less than -1, 1, etc., red blood cells

Chart 3.

Speed of Capillary Stream Before and After Operation

O = before > = after

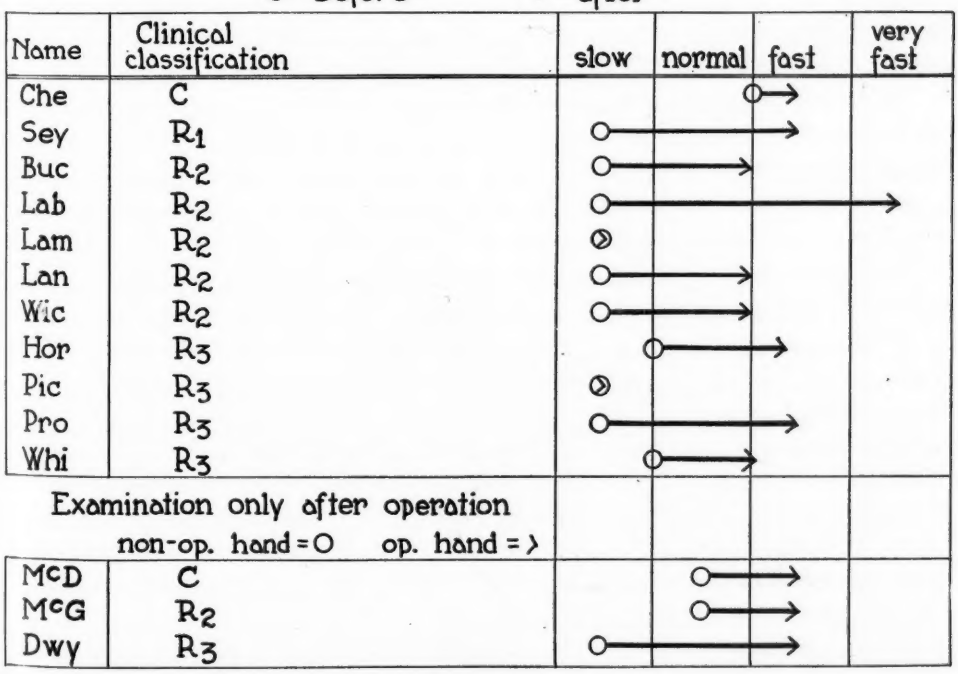


Chart 4.

and did not appear until after prolonged compression. This suggests a decrease in capillary permeability.

In the same patient it was also noted that the capillaroscopic picture, which had been very hazy before operation, became much clearer postoperatively. This was in obvious connection with the visible decrease in the swelling of the finger, i.e., with the decrease in the free fluid in the tissue. In addition, the dilatation of the excretory ducts of the sweat glands, which was present preoperatively, was no longer seen.

Capillaroscopic observations of sympathectomized Raynaud's disease have been published by Adson and Brown.[1] The authors discuss a case of Raynaud's disease which had a "resection of the sympathico-cervico-thoracic and second thoracic ganglion and the intervening trunk" on one side. They described the improved capillary flow, the brighter color of blood, and the narrowness of the capillaries on the operated side. The latter they attribute to increased tonus of the capillaries.

Change in the reflux time after operation varies, depending on its preoperative value and on the time which has elapsed since operation. In the group with decreased reflux time were cases with stases occurring after a few seconds as well as those with instantaneous stases. It was shown that the reflux time in these cases increases after operation. For example, in one patient (Syp), the reflux time before operation was only six seconds. On examination two weeks after operation the reflux time was seventy seconds, three weeks after operation, fifty-five seconds, and five weeks after operation, twenty-five seconds. After six weeks there was again a decrease in the reflux time, so that immediately after pumping up the blood pressure cuff there was a stasis in the capillary stream. Three months after operation the reflux time reached forty-five seconds, and six months afterward it was about the same, thirty-nine seconds.

In another patient (Whi) who showed a short reflux time of five seconds preoperatively, the same value was obtained on the fourth day after operation. On the fifth day the reflux time was 52 seconds, on the twelfth, fifteen seconds. However, this was observed three days after an operation on the other arm and the reflux time was fifteen seconds in both extremities.

In patient Che, who showed an immediate stasis upon compression before operation, the reflux time was eight seconds eleven days after operation.

In another patient (Lan), with a decreased reflux time on the right side to four seconds and on the left to five seconds, examination of the left hand five days after operation showed a reflux time of nine seconds, and sixteen days after operation, examination of the right hand showed a value of fifteen seconds.

Finally, in patient Lab, in whom, before operation, there was an immediate stasis of the capillary flow upon pumping up the cuff, the reflux time in the right arm was thirty-seven seconds one week after operation and eighteen seconds in the left arm two weeks after operation.

In some cases with a low reflux time no changes therein appeared during the first four days postoperatively, but the increase is first seen shortly thereafter.

This condition is particularly noteworthy because between the fourth and fifth days there is frequently also an unusual change in the appearance of the hand. Usually, during the first four days after operation, as Smithwick[31] has often emphasized, the hand is still cold and moist, while on the fifth day it becomes warm and dry.

The curve for the reflux time in the above-mentioned case (Syp), as in other cases, shows that the increase in time which appears a few days after operation gradually decreases in the course of the following few weeks, but that later a value is established which does not change in the following few months and seemingly may be regarded as constant.

The values of the reflux time obtained for patient Whi create the impression that the operation on one side influences the postoperative results on the other side, since the reflux time in one arm, increased because of the operation, decreases at the moment a sympathectomy is performed on the other side.

In summary, it may be said that in cases of Raynaud's disease with lowered reflux time there is an increase after operation.

In discussing those cases with an increased reflux time before operation, it must be stated that in the majority they are severe cases. Here we see the opposite of what we saw in cases with decreased reflux time. After operation there is a significant decrease in reflux time, the value sometimes sinking below normal. Patient Pic, who was examined several times before operation, showed a markedly increased reflux time in her enormously dilated capillaries. However, it was striking that before operation a stasis could occasionally be seen immediately following compression. This, however, was not always due to compression, but was one of the frequent spontaneous stases which were characteristic of this case. These spontaneous stases ceased after operation. Sixteen days after operation the reflux time was fifteen seconds. Two and one-half months after operation the afterflow time in the right arm was twenty-six seconds, in the left, three months after operation, it was five seconds.

Another patient (Hor) who, before operation, had a reflux time of ninty seconds in the right hand and of sixty-five seconds in the left hand, showed a reflux time of eight seconds on the left hand fifty-one hours after operation on this side, and of five seconds on the fourth day after operation. On examination two weeks after left-sided preganglionic dorsal sympathectomy and one week after right-sided preganglionic dorsal sympathectomy, the reflux time in the right hand was twenty-five seconds.

In patient Pro, who, according to the clinical picture and the capillaroscopic findings is one of the severe cases of Raynaud's disease, a reflux time of thirty-seven seconds was found before operation. Three days after left-sided preganglionic dorsal sympathectomy and twelve days after right-sided preganglionic dorsal sympathectomy the reflux time was thirty seconds on the left and thirty-five seconds on the right. Four days later, in the left hand, the reflux time was twelve seconds; in the right hand varying results were obtained in variously formed capillaries of different widths. In a narrow capillary a time of twelve seconds was found and in a widened giant capillary, thirty-two seconds, while in the deeper layers values of five seconds and eight seconds were obtained.

In general, it may be said that in cases with a pathologically increased reflux time, a sudden and excessive fall thereof occurs immediately after operation. Then its value gradually approaches a normal level.

In some patients with preoperatively prolonged reflux time in whom three or more months have passed since operation, findings of thirty-five to forty-five seconds reflux time have often been obtained, i.e., a moderately prolonged reflux time. This finding was obtained in 13 of 25 hands which have been examined to date. In few patients in whom there was no retrograde flow in the compression experiment, a reversal in the direction of flow was seen upon compression after operation.

The *critical capillary pressure* has a distinct relation to the systolic blood pressure. If the blood pressure cuff is pumped over the systolic pressure and kept at this level, no blood can flow through the arterioles and reappearance of the capillary flow is impossible. Those few cases which, paradoxically, have a critical capillary pressure above the initial systolic pressure can doubtlessly be explained by the fact that they have a markedly fluctuating blood pressure which rose above its initial value during the examination.

If we compare the preoperative and postoperative differences between systolic pressure and the critical capillary pressure, we almost always see that after operation the critical capillary pressure is nearer the systolic pressure than before operation. Of the 11 cases in which this observation was made before and after operation, 10 showed a decrease and one showed an increase (Chart 2).

An interesting phenomenon which we observed in several cases and which was clearly changed after operation is the following: A series of patients who bore the occlusion of the circulation in the compression experiment $1' - 3'$ without any symptoms, upon gradual release of the pressure, felt a distinctly painful sensation at about the level at which the stream reappeared capillaroscopically. After sympathectomy this pain was no longer elicited.

The number of capillaries per microscopic field visible at the nailfold before and after operation shows no marked variation. Our observations showed no increased number of capillaries such as described by others. In a few cases after operation the first row of visible capillaries was much further from the corium than before operation This can be explained by the fact that the capillaries of the first row are completely emptied after operation.

Before these findings can be discussed, the correlations between the speed of the capillary flow and the pulse rate should be considered. Such a comparison in our patients shows that there is no direct correlation, but, on the other hand, in cases with slow pulse rates, both rapid and slow rates of flow are found. This did not surprise us, since in patients who had a unilateral sympathectomy we found a sluggish capillary flow contralaterally and a more rapid flow homolaterally. In nonoperated persons with Raynaud's disease who had a rapid pulse rate, we almost always found a sluggish flow on the affected side. Those patients whom we observed with pulse rates over 100 were severe cases of the disease.

This phenomenon might be explained by supposing that the high pulse rate represents a compensatory mechanism to bring about a better flow. One might also bear in mind that, according to various authors, in cases of Raynaud's disease there is an increased activity of the adrenals, and this in turn causes the higher pulse rate. In any case, it shows that capillary circulation is, to a great extent, independent of the main circulation and probably reacts to processes in the tissue.

A simple relationship between systolic pressure and the rate of capillary flow does not exist, since we find rapid as well as sluggish flow in cases with normal pressure. This is apparent in those patients operated on on one side only, since they show a rapid flow on the operated side and a sluggish flow on the nonoperated side.

In the nonoperated patients it was striking that in the group of those with particularly low blood pressure (80 to 110 mm.) the capillary flow was slow in 7 of 9 cases in the group.

DISCUSSION

We can now explain the previously mentioned postoperative pallor and higher temperature of the hand. The former is due to the narrowness of the capillaries which is now present. The latter is due to the increased rate of flow following the operation. One might say, rather, that the temperature of the hand is dependent on the amount of blood flowing through the skin capillaries per unit time, and this is partly dependent on the width of the arteries.

One must not assume therefore, that the acceleration of the flow seen microscopically is a consequence of the small diameter of the capillaries, but, because of the warmth of the hand, one must maintain that despite the narrowness of the capillaries the amount of blood flowing through them at a given time is greater after sympathectomy than before it.

The pallor of the hand observed postoperatively agrees with the findings of Dale and Richards,[9] who made their observations on the unpigmented cat's paw and saw that the denervated extremity was distinctly warm, but at the same time paler than the pads of the normal foot.

The narrowing of the capillaries after sympathectomy occurs for various reasons. In describing capillary behavior we have avoided the use of the words "spastic" and "atonic" and have restricted ourselves to the words "narrowed" and "widened," in order to emphasize that the change in the width of the capillaries is not indicative of an active movement of the vessels. There is no doubt that the change in the width of the capillaries is not dependent to any great extent on the flow in the arterioles, but is for the most part the result of metabolic changes in the surrounding tissues. Dilatation of the arterial limb is due to a passive distention as a reaction to the environment and swelling of Rouget's cells, whereas narrowing is attributed to inhibition of the surrounding tissue which then compresses the capillaries. Dilatation of the venous limb is more the result of changes in the environment, i.e., of the production of acid metabolites. Since the capillaries have only endothelial layers, no smooth muscle and no connective tissue, the sympathectomy can affect only the arterioles. For this reason, the operation may help in part indirectly: (1) by elimination of the spasm of the arterioles bringing about an acceleration of the blood flow

and a consequent improvement in the local tissue metabolism; (2) through the liberation of a chemical substance (H-substance), the intermediary production of which was inhibited before the nerve fibers were dissected.

The improvement in the capillary condition and the narrowing, therefore, occurs because (a) after the elimination of the spasm the vis a tergo causes an increased rate of flow, (b) the content of the capillaries becomes more mobile, (c) the external pressure decreases due to the changed condition of the tissue, (d) the capillary wall becomes less permeable to the plasma, the blood becomes more dilute, the blood corpuscles less conglobated. Each of these factors plays a role.

In consideration of the above-mentioned factors, the postoperative change in the reflux time becomes significant as a functional test. In the cases with sluggish blood flow and very short reflux time, the operation produced an increase in the reflux time because of the reasons discussed, an acceleration of the blood flow, diminution of the tissue pressure, and dilution of the blood. In the few cases with a sluggish flow and a reflux time lengthened because of the tremendous expansion of the capillaries, the operation provoked a relative reduction of the reflux time, particularly by relaxing the arterial spasm and improving the blood flow.

The critical capillary pressure depends on the same factors which affect the reflux time. As a result of the sympathectomy, the critical capillary pressure is nearer the systolic blood pressure, varying directly with the effect of the operation on the basic pathologic factors. The more rapid the capillary flow and the narrower the capillaries, the closer to systolic pressure is the critical capillary pressure.

All these observations show the manifold pathways by which the damaging factors lead to the development of Raynaud's disease. Emotional factors may play a role in the precipitation and in the continuation of these processes, but further study is needed before the importance of this role can be evaluated.

SUMMARY

In this investigation the capillary response was studied in 29 cases of Raynaud's disease.

1. Capillaroscopic observation of cases of Raynaud's disease shows that the disease may be present before the appearance of the clinical symptoms. In these cases abnormal capillaries can be seen when other vasomotor symptoms are not present.

2. In the majority of cases of Raynaud's disease there exists a persistence of the subpapillary plexus and a persistent connection between it and the outgrowing capillaries.

3. Two objective measurements are introduced to test the dynamics of capillary circulation: (a) *reflux time,* i.e., that time which elapses before the capillary flow ceases, when the upper arm is compressed with a blood pressure cuff, pumped up to 30 mm. above the systolic blood pressure; (b) *critical capillary pressure,* i.e., that pressure at which the flow once again reappears when the cuff in the above experiment is gradually decompressed at the rate of 10 mm. per two seconds.

4. Capillaroscopy can define the severity of the disease more accurately than the gross clinical findings.

5. The preganglionic sympathectomy has a distinct influence on the capillary picture of Raynaud's disease: (a) in 12 of 14 cases the speed of the blood flow increased; (b) in cases with a slow reflux time, the time increased, and vice versa; (c) in 10 out of 15 cases the width of the capillaries decreased; (d) the capillary permeability decreased; (e) the dilatation of the excretory ducts of the sweat glands vanished after operation; (f) these changes in the capillary picture after sympathectomy become less distinct in time; (g) the subjective symptoms of the patient are to some extent independent of the clinical picture. Some have the same typical capillary picture as before operation, even though relieved of complaints. Others show objective improvement clinically, but retain their subjective complaints, because a neurosis may become apparent, which may have been a part of the picture from the beginning.

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THE EXCRETION OF GOLD FOLLOWING THE ADMINISTRATION OF GOLD SODIUM THIOMALATE IN RHEUMATOID ARTHRITIS*

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IT IS generally agreed by those who have had a large experience with gold therapy in rheumatoid arthritis that this form of treatment is followed by encouraging therapeutic results in a large proportion of patients. However, toxic reactions and untoward effects are so common and so serious that there is great urgency for an increase in our knowledge relative to the mode of action, toxicology, and excretion of gold compounds. We have previously reported[1] the marked bacteriostatic power of the serum against many common laboratory organisms which develops following the administration of gold sodium thiomalate. Similar observations were made after the administration of gold sodium thiosulfate and aurolsulfide. These effects may, but do not necessarily, throw some light on the mechanism by which beneficial results are obtained with gold therapy. We recognize that as yet the causative agent of rheumatoid arthritis has not been demonstrated, and that the disease has not been conclusively shown to be of infectious origin.

OBJECTIVE

Our objective here is to report studies on the urinary excretion of gold following the parenteral administration of gold sodium thiomalate. In view of the long duration of unfavorable reactions when they do occur, a special objective was to determine the length of time excretion through the urine con-

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tinued after administration was stopped. Less complete observations were made on the gold content of the synovial fluid, stool, and saliva. We were also interested in determining the amount of gold present in the whole blood and blood serum at varying intervals, and the relationship of these levels to the urinary excretion, but these latter studies will be reported subsequently. All such studies, when coordinated with the occurrence of beneficial effects and toxic reactions, should lead to a clarification of the technique of aurotherapy.

METHOD

The method we used for the determination of urinary gold was described by Pollard[2] in 1937, and by Jamieson and Watson in 1938.[3] Some modifications necessary to make the method applicable to the analyses of materials other than urine will be more completely described in a subsequent paper. Analysis of known samples by these methods resulted in practically 100 per cent recovery. The details observed in the collection of specimens are recorded under the separate experiments described below. Whenever possible the twenty-four-hour urine collections were started at 8:00 A.M., and the gold salt was given as near after 9:00 A.M. as possible. All patients studied were suffering from rheumatoid arthritis and the observations were made during routine treatment with gold sodium thiomalate administered parenterally in aqueous solution. As a rule, no other medication was given. The average dose of gold sodium thiomalate varied from 25 mg. once a week to 50 mg. every other day. The results of our analyses are recorded in terms of metallic gold. It is to be remembered that gold sodium thiomalate contains only 50 per cent metallic gold.

RESULTS

Experiment 1. O. J., female, aged 35 years, with rheumatoid arthritis, received weekly injections of gold sodium thiomalate after two control urinalyses had been done. These, of course, were negative for gold. Subsequently, urines were collected twenty-four hours before and twenty-four hours after the injection of the gold salt. Table I records our results.

TABLE I

URINARY EXCRETION OF GOLD FOLLOWING SUBCUTANEOUS INJECTIONS OF GOLD SODIUM THIOMALATE

CASE	DATE	ADMINISTRATION OF GOLD SALT (MG.)	VOLUME 24 HR. URINE (C.C.)	URINARY EXCRETION AS Au IN 24 HR. (MG.)
O. J., female, aged 35 years	Mar. 14-15		1,125	0
	20-21		925	0
	21-22	5	840	0.38
	27-28		1,000	0.08
	28-29	10	935	0.15
	April 3-4		1,125	0.08
	4-5	25.0	780	0.30
	10-11		860	0.13
	11-12	25.0	810	0.42
	17-18		720	0.23
	18-19	25.0	1,060	0.44
	24-25		1,000	0.18
	25-26	25.0	770	0.28
	May 1-2		800	0.24
	2-3	25.0	700	0.75
	8-9		510	0.32
	9-10	25.0	600	0.40

It will be observed that the urinary excretion of gold was less during the twenty-four hours before than the twenty-four hours after the injection of gold salt, but that this difference appeared to decrease week by week.

Experiment 2. A male, aged 38 years, was given injections up to 50 mg. every other day. It will be observed (Table II) that the urinary excretion gradually increased, reaching a maximum of 2.07 mg. During the thirteen days under observation he received a total of 280 mg. of gold salt (140 mg. of metallic gold). He excreted through the urine during this period (allowing an estimated excretion of 0.40 for June 15 to June 16, when the urinary determination was omitted) a total of 12.83 mg. of metallic gold, or roughly 9 per cent of his intake of metallic gold during this period. Serum levels were determined on four days, the results varying from 0.22 to 0.50 mg. per cent. On June 18 the serum content was 0.50 mg. per cent and the synovial fluid content was likewise 0.50 mg. per cent. For three days, starting June 18, carmine-marked stools were collected. It was found that during this three-day period, the patient excreted through the stool only 0.66 mg. It will be observed that the urinary loss during this three-day interval was 4.27 mg. The stool excretion, therefore, was very much less in proportion to the urinary excretion during these three days.

TABLE II

URINARY AND STOOL EXCRETION OF GOLD, AND GOLD CONTENT OF SERUM AND SYNOVIAL FLUID FOLLOWING SUBCUTANEOUS INJECTIONS OF GOLD SODIUM THIOMALATE

(Male, Aged 38 Years)

DATE	GOLD SALT (MG.)	VOLUME 24 HR. URINE (C.C.)	Au CONTENT 24 HR. URINE (MG.)	Au CONTENT SERUM (MG. %)	Au CONTENT STOOL (MG. %)	Au CONTENT SYNOVIAL FLUID (MG. %)
June 12-13	5	1,075	0.05			
13-14		910	0.08			
14-15	25	1,050	0.37			
15-16						
16-17	50	2,100	0.55			
17-18		1,200	0.90			
18-19	50	2,200	1.58	0.50		0.50
19-20		1,475	1.15		0.66	
20-21	50	1,600	1.54	0.32		
21-22		1,650	1.23			
22-23	50	1,800	1.67	0.22		
23-24		1,750	1.64			
24-25	50	1,700	2.07	0.44		

Experiment 3. M. W., female, aged 38 years, had received 330 mg. of gold sodium thiomalate previous to the excretion studies we made. Subsequently, 25 mg. of gold salt were given every seven days. Table III records the daily urinary excretion of metallic gold. In this instance, the excretion became relatively fixed, varying between 0.17 and 0.51 mg. in twenty-four hours. From these observations, one might suggest that there was a renal threshold for gold excretion, but the evidence of Experiment 2 (where the excretion gradually increased) tends to contradict this impression.

During the fourteen days from April 26 through May 10 this patient received 50 mg. of gold salt or 25 mg. of metallic gold. She excreted through the urine during this period (assigning an estimated average of 0.33 mg. for April 27 when the determination was omitted) a total of 4.35 mg. of gold, or 17.2 per cent of the intake. This proportion is larger than that found in Experiment 2 where the percentage of excretion was 9 per cent.

Experiment 4. It was important to know how long the urinary excretion of gold continued after the administration of gold salt had been discontinued. Table IV records the gold content of the urine at varying days from 60 to 300 after the cessation of therapy. In Case 5 there was 0.04 mg. of gold in the urine on the 210th day, 0.03 mg. on the 240th day, but no gold on the 270th day. In Case 2, 0.01 mg. was found on the 300th day. All observations made show that gold is still excreted long after the administration has been discontinued.

TABLE III

URINARY EXCRETION OF GOLD FOLLOWING SUBCUTANEOUS INJECTIONS OF GOLD SODIUM THIOMALATE

CASE	DATE	ADMINISTRATION OF GOLD SALT (MG.)	VOLUME 24 HR. URINE (C.C.)	URINARY EXCRETION AS Au IN 24 HR. (MG.)
M. W., female, aged 38 years	Dec. 20, 1939 to Apr. 26, 1940	330		
	Apr. 25-26		370	0.36
	26-27	25.0	300	0.51
	27-28		–	–
	28-29		690	0.24
	29-30		880	0.25
	30-May 1		900	0.17
	May 1-2		750	0.35
	2-3		870	0.34
	3-4	25.0	1250	0.24
	4-5		500	0.47
	5-6		250	0.42
	6-7		825	0.42
	7-8		800	0.36
	8-9		775	0.34
	9-10		560	0.24
	10	25.0		

TABLE IV

URINARY EXCRETION OF GOLD AFTER STOPPING THE ADMINISTRATION OF GOLD SODIUM THIOMALATE FROM 60 TO 300 DAYS

CASE	NO. DAYS SINCE LAST DOSE	LAST DOSE (MG.)	TOTAL GOLD SALT GIVEN	VOLUME 24 HR. URINE (C.C.)	URINARY GOLD EXCRETION (MG.)
1	60	25	340	1,500	0.13
2	300	10	340	1,200	0.01
3	102	10	493 (second course)	1,000	0.16
4	90	30	575	840	0.16
5	210	25	200	530	0.04
	240	25	200	1,200	0.03
	270	25	200	1,675	0.00
6	135	50	1,100	1,400	0.09
7	100	30	410	860	0.10
8	98	50	1,005	1,370	0.17
9	90	10	85	1,650	0.08
10	210	25	1,000	1,000	0.09

TABLE V

EXCRETION OF GOLD BY THE STOOL

CASE	TOTAL GOLD SALTS GIVEN (MG.)	LAST DOSE (MG.)	NO. DAYS SINCE LAST DOSE	NO. DAYS STOOL STUDIED	TOTAL STOOL EXCRETION Au (MG.)	AVERAGE DAILY URINE EXCRETION Au (MG.)	AVERAGE DAILY STOOL EXCRETION Au (MG.)
1	355	25	One	1	0.64	?	–
2	285	50	Second day	3	1.94	?	0.65
3	180	50	First and third days	3	0.66	1.42	0.22

TABLE VI

EXCRETION OF GOLD BY THE SALIVA

CASE	AMOUNT OF GOLD SALTS GIVEN (MG.)	LAST DOSE (MG.)	NO. DAYS SINCE LAST DOSE	VOLUME OF SALIVA (C.C.)	Au CONTENT (MG.)
1	480	50	2	100	0
2	500	50	3	56	0

TABLE VII

URINARY EXCRETION OF GOLD FOLLOWING SUBCUTANEOUS INJECTIONS OF GOLD SODIUM THIOMALATE, AND THE EFFECT OF SODIUM BICARBONATE ON THE URINARY EXCRETION

(Female aged 35 years)

DATE	GOLD SALT (MG.)	Au CONTENT 24 HR. URINE (MG.)	MEDICATION SODIUM BICARB. (DRAMS O.D.)
June 27	10		
28			
29	25		
30			
July 1	50		
2			
3	50		
4			
5			
6	50		
7			
8	50		
9			
10	50		
11			
12			
13	50		
14			
15	Slight dermatitis, both axillae, gold salt discontinued		
16			
17		0.65	
18		0.71	
19		0.96	
20		0.39	
21		0.58	
22		0.68	
23		0.50	
24		0.53	1
25		0.53	2
26		0.38	4
27		0.44	4
28		0.53	4
29		0.35	4
30			4

Experiment 5. A few observations were made on the metallic gold content of the stool. Stools were appropriately marked with carmine in each case. It will be observed (Table V) that the excretion of gold through the stool varied between 0.22 and 0.65 mg. in twenty-four hours.

Experiment 6. In two instances (Table VI) saliva was collected during the course of gold sodium thiomalate administration. In neither of these instances was metallic gold found in the saliva.

Experiment 7. S. P., female, aged 35 years, had never received gold before the institution of these observations. After she had received 330 mg. of gold salt, she developed a slight dermatitis under both axillae, which may or may not have been due to gold. However, the gold administration was discontinued. Subsequently from July 17 through July 29, the

daily urinary excretion was determined (Table VII). In this case, as in Experiment 3, the urinary excretion seemed rather fixed. After the seventh day of this relative fixation of excretion, we were interested in observing the effect of sodium bicarbonate on the rate of excretion. Up to 15.6 Gm. were administered by mouth daily. It will be observed that from the eighth to the thirteenth day, the urinary excretion continued relatively constant, the administration of sodium bicarbonate apparently having no effect in this respect.

Our object here was to observe whether sodium bicarbonate had the same accelerating effect on the excretion of gold as it has been found to have on the excretion of lead. These studies are being extended and will be reported more fully at a later date.

CONCLUSIONS

1. Following the subcutaneous administration of gold sodium thiomalate in patients with rheumatoid arthritis, metallic gold can be consistently recovered from the urine, and the twenty-four-hour excretion determined.

2. In two instances the urinary excretion of gold approximated 9 and 17.2 per cent of the intake of gold during the period of observation.

3. Gold was found in the urine from 60 to 300 days after administration of gold sodium thiomalate had been stopped.

4. The gold content of the stool in three instances ranged from 0.22 to 0.65 mg. in twenty-four hours.

5. No gold could be recovered from the saliva in the two instances studied.

6. As far as our observations were extended, the administration of sodium bicarbonate by mouth had no quantitative effect on the excretion of gold in the urine.

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STUDIES IN BACTERIOPHAGE VI

The Effect of Sulfapyridine and Sulfanilamide on Staphylococci and B. Coli and Their Respective Bacteriophages*

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INTRODUCTION

In a previous publication[1] we reported the effect of a large series of antiseptics, used in surgical practice, on staphylococcus, *E. coli*, and *B. pyocyaneus* bacteriophages. We found that sulfanilamide (prontosil and prontylin) and its derivative, sulfapyridine, were the only substances tested which exerted no significant influence on the lytic phenomenon.

In a number of later experiments with weaker phages, these drugs seemed to have an adjuvant effect upon the action of bacteriophage, but they had no appreciable effect on phages of high titer and lytic ability. This suggested that the combination of phage and one of these drugs may be used in those cases in which the causative organism was partially resistant to phage or in mixed infections where each may have a specific action.

EXPERIMENTAL TECHNIQUE

Our stock mixture of staphylococcus phages S1, and individual phages 43† and BH‡ were tested against 50 strains of staphylococci obtained from various sources and possessing various degrees of phage susceptibility, with and without sulfapyridine and sulfanilamide.

Our stock mixture of *E. coli* phages B4 and individual phage MK were likewise used in conjunction with these two substances when propagated against 45 strains of *E. coli* of various degrees of susceptibility.

The technique of the experiments was in general the same as in our previous work.[1] All lysis tests were carried out in 5.5 c.c. of Savita broth. Plating of lyzed tubes on 5 per cent sheep's blood agar indicated the presence or absence of surviving bacteria. The titer of the phages was determined by making decimal dilutions in saline, adding bacteria and then counting the plaques when these dilutions were transplanted and incubated on 1 per cent plain agar plates. Generally, in carrying out phage susceptibility tests, 0.05 c.c. of staphylococcus phage, or 0.25 c.c. of *E. coli* phage, were used in 5.5 c.c. of Savita broth, together with approximately 50 to 60 million bacteria per cubic centimeter. Variations from this technique are indicated in certain experiments.

Sulfapyridine and sulfanilamide were used in a 10^{-4} dilution (1:10,000), which is about the highest concentration which these substances ordinarily reach

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†Obtained in 1933 from Dr. Bulgakov.

‡Obtained in 1933 from Dr. Gratia.

in the circulation of treated patients. In order to obtain the necessary concentration, 0.5 c.c. of the 10^{-3} dilution (1:1,000) of these drugs in Savita broth (sterilized by boiling for five minutes) was added to 5 c.c. of Savita.

RESULTS

A. *The Effect of Sulfapyridine (SP) and Sulfanilamide (SN) on the Staphylococcus Bacteriophage Phenomenon*

I. *The Degree and Permanency of Bacteriolysis Both With and Without SP and SN.*—S1 phage (slightly weakened by forty-eight-hour standing at room temperature) was set up with 15 strains of staphylococci, most of which were somewhat resistant to the phage. Two series of tubes containing 5.5 c.c. of Savita plus sulfapyridine SP (1:10,000) were employed. One series was inoculated with 0.05 c.c. of phage and 0.1 c.c. of an eighteen-hour Savita broth culture of bacteria (making about 50 million per cubic centimeter), and the other series with bacteria alone. Two series of 5.5 c.c. of Savita without sulfapyridine, inoculated in a similar manner, served as controls. The results of the test are shown in Table I. It is seen that after four and a half hours' incuba-

TABLE I

		CULTURES														
	TIME	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Lysis by phage S1 in Savita	4½ hours	–	4+	4+	3+	3+	3+	–	4+	1+	4+	4+	4+	4±	4+	1+
	22½ hours	–	1+	4+	–	4+	4+	1+	4+	4+	4+	4+	4+	4+	4+	3+
	5 days	–	–	2+	2+	4+	4+	–	–	4+	4+	1+	4+	–	4+	2+
Lysis by phage S1 in Savita + SP	4½ hours	–	4+	4±	4+	4+	3+	1+	1+	2+	4+	4±	3+	4+	3+	1+
	22½ hours	–	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	5 days	–	4+	4±	4+	4+	4+	2+	4+	4+	4+	4+	4+	4+	4+	4±
Bacterial control in Savita	4½ hours	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	22½ hours	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Bacterial control in Savita + SP	4½ hours	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	22½ hours	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

4+ = Complete clearing.
4± to ± = Different degrees of clearing.
– = No lysis.

tion, the degree of clearing or growth in corresponding tubes with and without SP was essentially the same. After twenty-two and a half hours' incubation, four cultures, only partially clear in the absence of sulfapyridine, were completely clear in the corresponding tubes containing sulfapyridine. After five days' incubation five cultures which were cloudy in the absence of sulfapyridine were completely clear (4+) in the corresponding tubes containing sulfapyridine, and two others, which in the absence of SP showed 2+ lysis, were almost clear (4±).

The experiment was repeated with the individual phage No. 43 and eight strains of staphylococci, and similar results were obtained. This phage produced complete visible lysis of all these strains in the test tubes without SP or SN in twenty-four hours, but five of the cleared cultures yielded growth upon plating and these tubes became cloudy within seventy-two hours. The corresponding tubes containing phage and either SP or SN not only became clear, but also gave no growth upon plating, and remained clear for fifty days at room temperature when examined last.

In attempting to explain these results, the question naturally arose whether this effect was attained through an increase in the lytic power of the bacteriophage or whether it was due to some effect upon the bacteria. In the series of experiments which follow we have attempted to answer these questions.

II. *The Significance of Bacterial Concentration on the Bacteriolytic Phenomenon Both With and Without Sulfapyridine and Sulfanilamide.*— Staphylococcus phage mixture S1 and single phage BH were tested against four strains in amounts of 0.1 c.c. and 0.35 c.c., and 0.6 c.c. of an eighteen-hour Savita culture, which made suspensions of approximately 50 million, 175 million, and 300 million bacteria per cubic centimeter (Table II). It is evident that with both SP and SN the small bacterial inoculations gave complete clearing with phage with all strains, while the higher bacterial concentrations did not.

TABLE II

CULTURE	0.1 c.c.			0.35 c.c.			0.6 c.c.		
	Sav.	Sav. + SP	Sav. + SN	Sav.	Sav. + SP	Sav. + SN	Sav.	Sav. + SP	Sav. + SN
H									
4 hours	4+	4+	4+	4+	4+	4+	3+	3+	3+
24 hours	4+	4+	4+	4+	4+	4+	4+	4+	4+
6 days	–	4+	4+	–	4+	4+	–	4+	4+
D									
4 hours	4+	4+	4+	4+	4+	3+	2+	±	±
24 hours	4+	4+	4+	4+	4+	4+	4+	4+	4+
6 days	–	4+	4+	–	–	4+	–	–	–
Bu									
4 hours	1+	1+	1+	±	±	±	–	–	–
24 hours	4+	4+	4+	4+	4+	4+	2+	±	–
6 days	–	4+	4+	–	–	–	–	–	–
K									
4 hours	4+	4+	4+	–	2+	2+	1+	–	–
24 hours	4+	4+	4+	4+	4+	4+	2+	2+	4+
6 days	4+	4+	4+	–	4+	4+	–	–	–

4+ = Complete clearing and no growth.
4± to ± = Different degrees of clearing.
– = Normal growth.

III. *The Effect of Sulfapyridine and Sulfanilamide Upon the Titer and Lytic Power of Staphylococcus Bacteriophage.*—

1. *The effect after a single twenty-four-hour passage as compared with five subsequent twenty-four-hour passages:* S1 phage was propagated on nine different strains, and No. 43 phage on three of these nine. After one passage seven of nine showed no appreciable difference between the titers of the Savita broth as compared with Savita plus sulfapyridine. In two, however, there was an appreciable lowering of titer in the presence of the drug. After five passages there was no increase or decrease in the titer or lytic ability of these phages, and no appreciable difference between the Savita phage and those containing SP and SN.

2. *The effect of storage in the refrigerator after a single passage:* Bacteriophages S1 and 43 were propagated at the expense of nine and three strains, respectively, in Savita broth both with and without SP or SN. They were then filtered and placed in the refrigerator. Titrations were carried out immediately, and again after six days, ten days, and one month. The titer was essentially

unchanged throughout this period both with and without SP and SN, except for two phages with SP, which were of definitely lower titer after one passage and remained at this titer throughout.

3. *The effect of SP and SN on the adaptation of staphylococcus bacteriophage to partially or completely resistant staphylococcus strains:* Six strains of staphylococci, four of which were partially resistant and two completely resistant to phage, were used for the repeated propagation of phage in Savita containing sulfapyridine and sulfanilamide. The propagated phages were then applied to the original cultures, and it was found that their lytic power had not increased.

IV. *The Effect of Sulfapyridine and Sulfanilamide on Staphylococci in the Absence of Bacteriophage.—*

1. *The effect on five consecutive twenty-four-hour passages in Savita both with and without sulfapyridine and sulfanilamide:* In this experiment, 0.1 c.c. and 0.01 c.c. of an eighteen-hour culture of eight strains of *Staphylococcus aureus* (three susceptible, two partially susceptible, and three resistant to phage) were inoculated into tubes containing 5.5 c.c. of Savita, Savita plus SP, and Savita plus SN, respectively, and incubated for twenty-four hours. One-tenth cubic centimeter of these cultures was then transplanted into similar media on four successive days. Readings were made after three, six, and twenty-four hours of incubation, and the clear tubes were plated for possible growth. Results of the effect of SP and SN on the tubes inoculated with 0.01 c.c. of these eight cultures and their four subcultures in respective media are presented in Table III. It is seen that all three strains which were completely resistant to phage were immediately inhibited by both SP and SN, and two of these were completely destroyed by SP in the course of the passages. On the other hand, the five strains of staphylococci which were completely or partially susceptible to phage, suffered only a transitory inhibition by SP and were practically not affected by SN.

2. *The effect of one twenty-four-hour passage on varying dilutions of bacteria:* In an attempt to study the action of SP and SN (dilution 1:10,000) on *Staphylococcus aureus* more closely, 0.1 c.c. of dilutions 10^{-2} and 10^{-4} of eighteen-hour cultures were taken beside the dilutions made with 0.1 c.c. and 0.01 c.c. of undiluted culture. In order to ascertain the number of bacteria per cubic centimeter of the suspension and its increase or decrease after contact with the drugs, a loopful (approximately 0.01 c.c.) of the highest dilution was plated immediately, and again after five and twenty-four hours of incubation. Growth in liquid media was followed after five, twenty-four, and forty-eight hours, and the clear tubes containing SP and SN were observed during two weeks of incubation. Fifteen strains of staphylococci, of which five were susceptible to phage, seven partially susceptible, and three entirely resistant, were used. Representative results of these experiments are presented in Table IV. An immediate plating of 0.01 c.c. of all the tubes which had been inoculated with 0.1 c.c. of the 10^{-4} dilution of eighteen-hour cultures of all 15 strains showed two to 34 colonies. After five hours of incubation similar transplants showed that the number of colonies on all plates increased considerably. However, in the presence of SP there were only one-half to one-fourth as many colonies as in plain Savita. After

TABLE III

	CU (SU)			O (SU)			PA (SU)			BU (PS)		
	SAV.	SP	SN	SAV.	SP	SN	SAV.	SP	SN	SAV.	SP	SN
Passage 1	++++	++++	++++	++++	++++	++++	++++	±	±	++++	++++	++++
Plating	0	0	0	0	0	0	++++	IC	IC	0	0	0
Passage 2	++++	–	++++	++++	+	++++	++++	++++	++++	++++	–	++++
Plating	0	IC	0	++++	27	0	0	0	0	0	40	0
Passage 3	++++	±	++++	++++	++++	++++	++++	++++	++++	++++	–	++++
Plating	0	IC	0	0	0	0	0	0	0	0	210	0
Passage 4	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+	++++
Plating	0	0	0	0	0	0	0	0	0	IC	IC	++++
Passage 5	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Plating	0	0	0	0	0	0	0	0	0	0	0	0
	K (PS)			PI (RE)			MN (RE)			KR (RE)		
Passage 1	++++	–	++++	++++	–	±	++++	–	±	++++	–	±
Plating	0	203	0	++++	–	±	++++	6	IC	++++	–	IC
Passage 2	++++	–	++++	++++	–	–	++++	–	++++	++++	–	++++
Plating	0	IC	0	++++	–	–	++++	–	++++	++++	1	0
Passage 3	++++	++++	++++	++++	–	–	++++	–	++++	++++	–	++++
Plating	0	0	0	++++	–	–	++++	–	++++	++++	–	++++
Passage 4	++++	++++	++++	++++	–	–	++++	+	++++	++++	–	++++
Plating	0	0	0	IC	–	–	IC	IC	IC	IC	1	++++
Passage 5	++++	++++	++++	++++	–	–	++++	–	++++	++++	–	++++
Plating	0	0	0	IC	–	–	IC	44	0	IC	–	++++

– = No visible growth; no growth on plating.
± to + = Various degrees of growth; ± showing hardly visible growth.
++++ = Normal growth.
IC = Innumerable colonies.
203 and other numbers = Number of colonies.
0 = Not plated.
SU = Susceptible.
PS = Partially susceptible.
RE = Resistant.

TABLE IV

TABLE IV

EFFECT OF 10^{-4} DILUTION OF SULFAPYRIDINE AND SULFANILAMIDE ON VARIOUS AMOUNTS OF STAPHYLOCOCCUS AUREUS IN SAVITA BROTH

	0.1 c.c. UNDILUTED CULTURE			0.1 c.c. DILUTION 10^{-1}			0.1 c.c. DILUTION 10^{-2}			0.1 c.c. DILUTION 10^{-4}		
	SAV.	SP SAV.	SN SAV.	SAV.	SP SAV.	SN SAV.	SAV.	SP SAV.	SN SAV.	SAV.	SP SAV.	SN SAV.
Culture O Susceptible to Phage												
Original colony count										9	5	5
Growth—5 hours	++++	++++	++++	++++	++++	++++	++++	–	++++	++++	–	+
Colony count—5 hours										65	13	3
Growth—24 hours	++++	++++	++++	++++	++++	++++	++++	–	++++	++++	–	++++
Colony count—24 hours							++++	IC	++++	++++	70	++++
Growth—48 hours	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+	++++
Culture Au Partially Susceptible to Phage												
Original colony count										15	9	10
Growth—5 hours	++++	++++	++++	++++	++++	++++	++++	–	+	++++	–	–
Colony count—5 hours										529	179	233
Growth—24 hours	++++	++++	++++	++++	++++	++++	++++	–	++++	++++	–	++
Colony count—24 hours							++++	IC	++++	++++	200	++++
Growth—48 hours	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	–*	++++
Culture Ku Partially Susceptible to Phage												
Original colony count										31	22	34
Growth—5 hours	++++	++++	++++	++++	++++	++++	++++	–	+	++++	±	±
Colony count—5 hours										1396	267	429
Growth—24 hours	++++	++++	++++	++++	++++	++++	++++	–	++	++++	–	–
Colony count—24 hours								IC		++++	28	IC
Growth—48 hours	++++	++++	++++	++++	++++	++++	++++	++	++++	++++	–*	++
Culture Ad Resistant to Phage												
Original colony count										29	24	29
Growth—5 hours	++++	++++	++++	++++	–	+++	++++	–	–	+++	++	±
Colony count—5 hours										555	146	93
Growth—24 hours	++++	++++	++++	++++	–	+++	++++	–	–	++++	–	–
Colony count—24 hours	++++	++++	++++	++++	IC	IC	++++	200	IC	++++	–	10
Growth—48 hours	++++	++++	++++	++++			++++	–*	++++	++++	–	–*

Colony count was made by spreading upon a blood plate of a loopful (0.01 c.c.) of 5.5 c.c. Savita tube inoculated with 0.1 c.c. of 10^{-2} and 10^{-4} dilutition of eighteen-hour Savita culture.

– = No growth in Savita and on plating.

± to +++ = Various degrees of growth in Savita.

++++ = Normal growth in Savita and on plating.

* = Growth appeared after several days of incubation.

IC = Innumerable colonies.

twenty-four hours of incubation the 10^{-4} dilution of each of the 15 cultures grown in SP displayed no visible growth. Upon further incubation it was noted that all but three grew eventually. Of these three destroyed strains, two were resistant to phage and the third was phage susceptible. All three strains when inoculated in 0.01 c.c. amounts of undiluted culture were again the only ones which gave no visible growth in SP tubes.

The effect of SN, although generally lower, was most pronounced in regard to the same three strains.

Out of seven strains used in all of the experiments, which were completely resistant to phage, six proved to be most susceptible to the bacteriostatic and bactericidal effect of SP and SN.

Of all 15 strains susceptible and partially susceptible to phage, only one showed a susceptibility to SP and SN similar to that of phage-resistant strains. Although the others were always affected by SP, they were destroyed by this drug only when they were in high dilution. When the bacteria were not destroyed within twenty-four hours, visible growth always developed upon further incubation.

V. *Morphologic Changes Under the Action of Sulfapyridine and Sulfanilamide.*—SP and SN not only inhibited the growth of certain strains in broth, but also altered to some extent the appearance of colonies when subcultured on a blood agar plate. Changes in size, shape, pigment production, and hemolysis were observed. Some colonies were degenerated and indented or pin point in size, presenting the appearance similar to changes after contact with bacteriophage. Some were normal yellowish, some were white, others were semitransparent, and still others were bright yellow. Some colonies were normally hemolytic, others were nonhemolytic, while a few were surrounded by a large zone of hemolysis. One culture treated with SN showed colonies surrounded by a double zone of hemolysis. Microscopically, the cultures grown in SP and SN showed the following morphologic changes: The size of some of the cocci was increased for two to six times. They were not as sharply outlined, the staining was less deep, and some seemed to be encapsulated.

VI. Effect of SP and SN on Phage Susceptibility of Staphylococci.—The phage susceptibility test on six strains of *Staphylococcus aureus,* which was carried out after twenty-four and forty-eight hours' contact of the bacteria with SP and SN at 37° C., showed no change in phage susceptibility of any of the strains. When the susceptibility tests were carried out after five days' contact with SP and SN, the results were inconclusive. Some strains became more resistant and others became more susceptible. Constant results could not be obtained.

B. *The Effect of Sulfapyridine (SP) and Sulfanilamide (SN) Upon the E. coli Bacteriophage Phenomenon*

I. *The Degree and Permanency of Bacteriolysis Both With and Without SP and SN.*—The effect of SP and SN upon *E. coli* and *E. coli* phages was studied along the same lines as the experiments with staphylococci. Table V presents results of the susceptibility tests of *E. coli* phage mixture B4 against ten strains of *E. coli* carried out with 0.1 c.c. of an eighteen-hour culture and 0.25 c.c. of

phage in 5.5 c.c. of Savita broth with and without SP and SN (dilution of 1:10,000). The effect of SP and SN upon the bacteria, in the absence of phage, was noted at the same time. From this table one can see that phage in Savita alone cleared seven of ten cultures within four hours, but only three maintained visible clearing for twenty-four hours, and two of these developed growth upon further incubation. The same phage set in Savita, plus SP, produced complete clearing after four hours of the same seven strains, and maintained it for twenty-four hours. Four of these strains maintained lysis for a week in the incubator, while three became cloudy. Two other cultures, which were partially cleared after four hours and remained so after twenty-four hours, later became a little more cloudy in the presence of SP. One of the ten cultures was completely resistant to phage and failed to undergo any lysis even in the presence of phage and SP. Contrary to the results obtained with staphylococci, the addition of SN to *E. coli* phage-bacteria mixtures had much less effect than SP.

TABLE V

	LYSIS READING	CULTURES 1	2	3	4	5	6	7	8	9	10
Phage B4 in Savita	After 4 hours	4+	2+	4+	2+	4+	4+	4+	–	4+	4+
	After 24 hours	–	3+	4+	2+	–	4+	4+	–	–	–
	After 1 week	–	–	4+	–	–	2+	1+	–	–	–
Phage B4 in Savita + SP	After 4 hours	4+	2+	4+	3+	4+	4+	4+	–	4+	4+
	After 24 hours	4+	4+	4+	3+	4+	4+	4+	–	4+	4+
	After 1 week	–	3+	4+	1+	4+	4+	4+	–	1+	2±
Phage B4 in Savita + SN	After 4 hours	4+	2+	4+	2+	4+	4+	4+	–	4+	4+
	After 24 hours	–	3+	4+	3+	±	4+	4+	–	–	–
	After 1 week	–	–	4+	–	–	4+	–	–	–	–
Controls											
Plain Savita	After 4 hours	–	–	–	–	–	–	–	–	–	–
	After 24 hours	–	–	–	–	–	–	–	–	–	–
Savita + SP	After 4 hours	1+	2+	1+	2+	2+	2+	2+	±	1+	±
	After 24 hours	1+	1+	1+	±	±	1+	1+	–	1+	1+
Savita + SN	After 4 hours	1+	1+	±	1+	1+	1+	–	–	±	–
	After 24 hours	±	±	±	–	–	±	±	–	–	–

4+ = Complete lysis.
3+ to 1+ = Various degrees of lysis.
± = Doubtful.
– = No lysis or normal growth.

Table V also shows that the SP alone produced a marked bacteriostatic effect upon most of the strains. The effect of SN was less than SP both in degree and extent.

The experiment was repeated with 35 more strains of varying resistance to phage, and similar results were noted. It was found that there was a definite correlation between the potency of phage against a given strain and the degree and duration of lysis attained by phage upon addition of SP. The cultures which were not completely cleared by phage in plain Savita within eight hours, showed, in Savita plus SP, a greater degree of lysis, but still it was not complete. The cultures which were completely cleared by phage in plain Savita within eight hours, but by twenty-four hours showed a secondary growth, were maintained clear in SP tubes for a period of twenty-four hours or longer. In other words, the SP prevented or delayed secondary growth.

Furthermore, the cultures that remained clear in Savita for at least twenty-four hours but gave growth upon plating on blood agar, were completely destroyed in the presence of SP. The strains that were permanently destroyed by phage in Savita were, of course, in no way affected by the addition of SP.

II. *The Effect of Sulfapyridine and Sulfanilamide on the Titer, Lytic Ability, and Range of Activity of E. coli Phages.*—Bacteriophage mixture B4 and the individual bacteriophage MK were employed in these experiments and were propagated at the expense of three different strains. All these strains were susceptible to phage B4, but only two of the three were susceptible to phage MK, the third being partially susceptible. The titrations were carried out after one and after five twenty-four-hour passages. At the same time, the first group of phages, incubated for twenty-four hours in the presence of SP, SN, and in plain Savita, were placed in the refrigerator and were titrated after five days, two weeks, one month, and six months. When titrated after twenty-four hours' contact with SP and SN, both B4 and MK phages, propagated on three susceptible strains, suffered no drop of titer, but in the presence of one of these drugs increased tenfold instead of one hundred times as did the control. However, the effect of SP was quite pronounced when bacteriophage MK was propagated at the expense of the partially susceptible strain. In this instance, while the titer of the bacteriophage was lowered to 10^4 in Savita and Savita plus SN, phage developed normally and reached a titer of 10^7 in the presence of SP. This experiment was repeated with the same results, and in general confirmed similar findings obtained with staphylococcus bacteriophage when these were propagated on the partially susceptible strains.

The titration of B4 and MK after five twenty-four-hour passages showed no significant differences in the titer of the phages, whether propagated on any of the three strains in SP, SN, or plain Savita. There was also no significant decrease in the original titer after two weeks, or after one month of contact with the SP and SN in the refrigerator at 4° C. A slight decrease of the titer of phage was occasionally noticed after six months of contact. These results fully agreed with similar tests with staphylococcus bacteriophages.

The lytic ability and the range of activity of *E. coli* phages was usually unaffected by contact with SP, and only when phage was propagated on partially susceptible strains was the phage with SP slightly higher than its Savita control.

Innumerable attempts to increase the lytic ability of *B. coli* phages to partially susceptible strains by adding SP ended in complete failure.

III. *The Effect of Sulfapyridine and Sulfanilamide on E. coli Strains and Their Subsequent Susceptibility to Bacteriophage.*—The same technique was used as with staphylococcus cultures. In no case was there complete destruction of the strain in the course of several passages when the undiluted culture was used and there was no significant difference between the phage-susceptible and phage-resistant strains in their degree of sensitivity to the effect of SP. The effect of SN was even less than that of SP.

On the other hand, when the experiments were carried out with an inoculation of 0.1 c.c. of undiluted bacterial culture and compared with the 10^{-2} and 10^{-4}

dilutions of the bacterial cultures, it was found that SP occasionally destroyed the two higher dilutions, but did not significantly affect the undiluted cultures (see Table VI). The undiluted cultures were in general more susceptible to the *bacteriostatic* action of SP than the staphylococci, but the higher dilutions were less susceptible to the *bactericidal* action of SP than the staphylococci. The effect of SN again ran parallel with SP but was always less pronounced.

TABLE VI

EFFECT OF 10^{-4} DILUTION OF SP AND SN ON VARIOUS AMOUNTS OF *B. coli* IN SAVITA BROTH IN THE ABSENCE OF BACTERIOPHAGE

CULTURE	0.1 C.C. OF UNDILUTED CULTURE			0.1 C.C. IN DILUTION 10^2			0.1 C.C. IN DILUTION 10^4		
	SAV.	SP+ SAV.	SN+ SAV.	SAV.	SP+ SAV.	SN+ SAV.	SAV.	SP+ SAV.	SN+ SAV.
Culture I (Phage Susceptible)									
Immediate plating							8	5	6
4-hour plating							200	26	55
24-hour plating							++++	−	++++
4-hour Savita reading	++	+	+	±	−	−	−	−	−
24-hour Savita reading	++++	+++	+++	++++	−	+	++++	−	++
48-hour Savita reading	++++	++++	++++	+++	−	++++	++++	−	++++
Culture D (Phage Partially Susceptible)									
Immediate plating							3	.7	12
4-hour plating							++	++	++
24-hour plating							++++	++	++++
4-hour Savita reading	++++	+++	++++	+	±	+	−	−	−
24-hour Savita reading	++++	+++	++++	++++	+++	++++	++++	−	++++
48-hour Savita reading	++++	++++	++++	++++	++++	++++	++++	++	++++
Culture M (Phage Resistant)									
Immediate plating							13	5	12
4-hour plating							250	135	200
24-hour plating							++++	++++	++++
4-hour Savita reading	++++	++++	++++	+	±	±	−	−	−
24-hour Savita reading	++++	+++	++++	++++	+++	++++	++++	+	++++
48-hour Savita reading	++++	++++	++++	++++	++++	++++	++++	++++	++++

Colony count was made by spreading approximately 0.01 c.c. of the tube containing 0.1 c.c. of dilution 10^{-4} of eighteen-hour culture in 5.5 c.c. of Savita, Savita plus SP, and Savita plus SN upon a quadrant of blood plate.

++++ = Normal growth on plating or in Savita.

− = No growth on plating or Savita.

+++ to ± = Various amounts of growth on plating and Savita, ± being the smallest and +++ the most abundant.

Neither SP nor SN produced any significant morphologic changes in regard to size, form, or other characteristics of the colonies of *E. coli* or significant microscopic morphologic changes in the organisms themselves.

E. coli strains, which were subcultured or maintained up to five days in the incubator in the presence of SP and SN, showed practically no change in their susceptibility to phage.

C. *The Effect of Various Concentrations of Sulfapyridine Upon the Bacteriophage Phenomenon*

In order to ascertain whether concentrations other than 1:10,000 (10 mg. per cent) of SP (which bacteriophage might encounter in the body) exerted a similar effect upon bacteriophage and bacteria, dilutions of 1:1,000, 1:50,000, 1:200,000, and 1:500,000 were studied. The action of these dilutions of SP upon *E. coli* and staphylococcus cultures was tested simultaneously and compared with the dilution 1:10,000. This was carried out with eight strains of each species in the presence and absence of bacteriophage. The experiments showed that SP dilution 1:10,000, in conjunction with bacteriophage, was somewhat more effective than 1:1,000 or 1:5,000 dilutions. The dilutions 1:200,000 and 1:500,000, on the other hand, had absolutely no effect upon the bacteriophage phenomenon.

SUMMARY

We are reporting herewith a study of the effect of sulfapyridine and sulfanilamide upon staphylococcus and *E. coli* cultures and their respective phages with regard to the lytic phenomenon. This study was carried out with three staphylococcus phages against 50 strains of staphylococci and two *E. coli*, phages, together with 45 strains of *E. coli*.

1. The addition of sulfapyridine in the dilution 1:10,000 to staphylococcus and *E. coli* phage suspensions with partially susceptible bacterial strains definitely increased the bacteriolysis and often resulted in complete destruction of all bacteria. The best results were attained with bacteriophages capable of maintaining lysis for at least eighteen to twenty-four hours, and with an initial bacterial inoculation not exceeding 50 million bacteria per cubic centimeter.

2. The use of sulfapyridine with weaker phages, which maintained lysis for less than eighteen hours with 50 million bacteria per cubic centimeter without the drugs, usually resulted in prolongation of lysis up to several days, but complete sterilization was seldom attained.

3. Dilution 1:10,000 (10 mg. per cent) of sulfapyridine produced the most favorable effect upon the bacteriophage phenomenon. The effect of dilution 1:1,000 and 1:50,000 was slightly below that of 1:10,000 SP dilution. Dilutions 1:200,000 and 1:500,000, however, did not exert any effect upon the bacteriophage phenomenon.

4. Both sulfapyridine and sulfanilamide exerted a bacteriostatic as well as a bactericidal effect on *Staphylococcus aureus* and were capable of destroying dilute bacterial suspensions, usually not exceeding 5,000 per cubic centimeter. The effect of SP was much greater than that of SN, and this was further shown by its pronounced activity against higher bacterial concentrations. The rapidity, duration, and degree of bacteriostatic and bactericidal effect varied for each strain and was most pronounced and complete against six of seven phage-resistant strains.

5. Both sulfapyridine and sulfanilamide were capable of producing macroscopic morphologic changes with regard to the size, shape, pigmentation, and hemolytic properties of the colonies of staphylococci and also certain microscopic changes on the organisms themselves.

6. Both SP and SN produced some inconsistent changes in the state of phage susceptibility of completely and partially phage-susceptible strains, whereas they had no effect on the state of phage resistance of completely phage-resistant strains.

7. Sulfapyridine possessed a pronounced bacteriostatic and bactericidal effect upon *E. coli*, and was capable of destroying a suspension not exceeding 5,000 bacteria per cubic centimeter of phage-susceptible and partially susceptible strains. When confronted with heavy suspensions of these strains, its bacteriostatic effect was usually more pronounced than on similar suspensions of staphylococci; however, its bactericidal power on *E. coli* was less than on staphylococci.

8. Sulfanilamide caused no significant change in the degree of bacterial lysis of *E. coli* strains with phage, and had no significant bacteriostatic or bactericidal effect on *E. coli* cultures.

9. Sulfapyridine and sulfanilamide added for the first time to staphylococcus and *E. coli* phages propagated on susceptible bacteria occasionally decreased the bacteriophage titer (not more than ten times) and its lytic power. However, upon repeated passages or prolonged contacts the titer and lytic activity of phage usually were re-established to their original level. Preservation of phage in 1:10,000 dilution of SP for six months did not significantly change its original titer. However, the adaptation of phage to partially susceptible strains was more successful in the absence of SP, indicating that SP prevented an increase of potency of phages.

CONCLUSIONS

1. Sulfanilamide and sulfapyridine do not interfere significantly with the lytic action of specific bacteriophage on *Staphylococcus aureus* or *B. coli* in vitro when these organisms are completely susceptible to the corresponding phage.

2. With strains of these organisms, which are not completely destroyed by the lytic action of the corresponding bacteriophage, the associated use of sulfapyridine, and to a less extent sulfanilamide, frequently completes the destruction of the bacteria in vitro—a synergistic effect.

3. Sulfanilamide or sulfapyridine may be used together with specific bacteriophage in the treatment of staphylococcus or *B. coli* infections. There may be a synergistic effect with these combinations in vivo.

4. Bacteriophage is generally more bactericidal on the corresponding staphylococci and *E. coli* than either sulfanilamide or sulfapyridine.

5. In the treatment of staphylococcus and *E. coli* septicemia, the use of potent bacteriophage, when it is available, should not be delayed because of the attempted use of sulfanilamide or sulfapyridine.

We wish to acknowledge the technical assistance of Miss Olga Mordvin and Mr. John T. Goodner.

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VARIATION OF THE ORGANIZED SEDIMENT FOLLOWING PYRIDIUM ADMINISTRATION IN URINARY INFECTIONS*

A Technique for the Study of the Organized Sediment Volume

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IN THE papers by Reynolds, Wilkey, and Choy,[4] and Morrissey and Spinelli,[3] the effect of phenylazo-alpha-alpha-diamino-pyridine monohydrochloride† on the symptomatology of urinary infections was adequately evaluated. This study is restricted to observations of the effect of pyridium on the variation in the amount of the organized urinary sediment. Although it was undertaken primarily for the purpose of sediment study, observations as to burning, dysuria, and nocturia were also carefully noted in an attempt to correlate the reduction of the urinary sediment with a decrease in the severity of these symptoms.

The terms "organized" and "unorganized" sediment are used as defined by Hawk and Bergeim.[1] In this paper, granular, epithelial, blood, and other pathologic casts will not be a part of the organized sediment. The volume of the organized sediment was selected for study because the total sediment, which may consist to a great extent of the unorganized sediment, bears no direct relationship to the severity of the infection. In a twenty-four-hour urine collection the amount of the unorganized sediment is a function of the diet, the pH, and the external temperature. The amount of unorganized sediment is also a function of the urease enzyme of the bacteria, which acts at various rates, depending upon the amount of urease, the amount of urea, the amount of buffer phosphates, the ionic strength of the solution, the pH, and the temperature. Thus a voluminous precipitate of salts may occur even if only small amounts of pus cells, epithelial cells, and bacteria are present. Since the unorganized sediment precipitate depends upon many factors apart from the infection, it is unsatisfactory material to work with if one is interested in studying the effect of a drug in clearing up infections. Therefore, in this report, the aim is to exclude, as much as possible, the effect of unorganized sediment from the determinations of the organized sediment volume.

While this work was in progress the paper of Morrissey and Spinelli[3] was published in which tentative studies of sediment variation following pyridium administration were made. No mention was made as to control or technique of their sediment studies. They report "a reduction of one-half to two-thirds of the amount of urinary sediment in operative cases when the drug was used." Although pyridium was given to these patients preoperatively and postopera-

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tively, it is not specified whether the sediment studies were made preoperatively, postoperatively, or both. Since it is stated that these patients were "operative," we assume that postoperative determinations of sediment were made.

Obviously, sediment studies made soon after an operation are comparatively less significant, since the operation is designed to relieve the primary cause from which the infection developed. Thus the condition should clear up spontaneously. Although the drug may do it faster, it is impossible to separate the factor of spontaneous resolution due to better drainage from that of the action of the drug. Only those cases in which infections persist for months after operation were considered suitable for the sediment studies reported here.

In selecting material for this study, the following postulates were established and the choice of patients were so made:

1. Low nonprotein nitrogen. Patients with a high nonprotein nitrogen or those who show extensive kidney damage cannot properly eliminate the educt.*
2. Demonstrable pus cells in a fresh urine specimen—6 to loaded per high-power field.
3. No granular, epithelial, or blood casts, and not more than a trace to 1+ of albumin. These findings, if present, will give false values to the sediment determinations.
4. No gross hematuria, the variation of which from day to day gives sediment values that may have no relation to the severity of the infection.
5. Female patients only can be used:
   (a) Those who have only a small number of vaginal epithelial cells in the urine during the nonmenstrual period.
   (b) Those past the menopause.
   (c) Those who would cooperate in reporting menstrual cycle, so that determinations would not be made during that period.
6. If the patient is a postoperative case, sediment studies should not be started unless the infection persists one or two months after the operation for reasons given in the discussion by Morrissey and Spinelli.[3]
7. Patients who are being sounded cannot be used for sediment studies, since soundings injure the tissues and increase sediment.

PROCEDURE

The twenty-four-hour urines were collected in brown bottles of from 2 to 4 liter capacity, depending upon the output of the patient. Five cubic centimeters of a 2 per cent alcoholic solution of thymol were added, and the bottle was rotated so that all of its interior was coated by the solution. This is preferable to adding crystals, since the crystals interfere with the reading of the sediment in the calibrated end of the centrifuge bottle. The daily urine volume was measured, so that results could be expressed in both sediment per 100 c.c. and total sediment. A preliminary twenty-four-hour collection without pyridium

*Pyridium elimination product.

was tested for organized sediment by the process described below. For approximately two weeks sediment determinations were run on the subsequent twenty-four-hour periods. This was done in the same manner as that of the preliminary specimen, using pyridium in dosages of 600 to 900 mg. per day.

The twenty-four-hour specimen was well shaken and a portion of about 150 c.c. was placed in a beaker. The pH was adjusted to 5.5± 0.2 using 1:1 hydrochloric acid or 1:1 ammonium hydroxide solution. Nitrazine paper was used as the indicator. The pH of the urine was checked with a quinhydrone electrode, and the results agreed very well with the nitrazine paper.

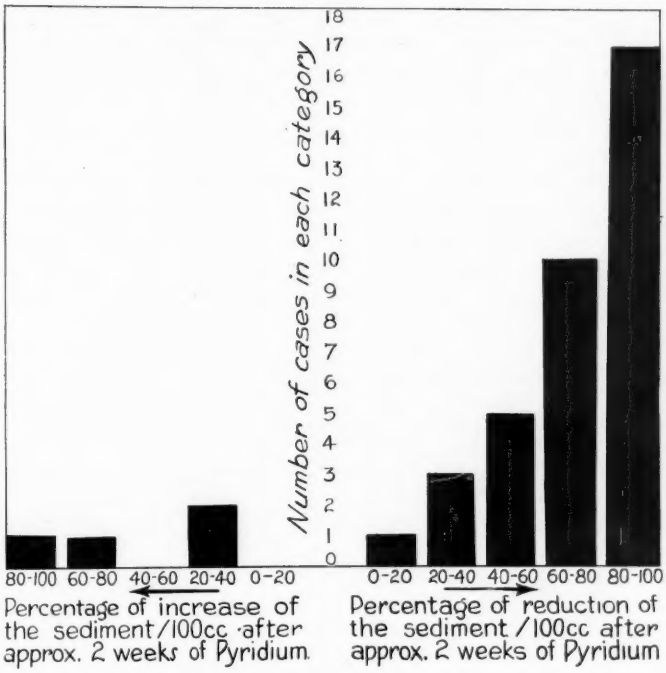
In the case in which the twenty-four-hour urine was at a pH of 5.5 or lower, an excess of ammonium hydroxide was added until a pH of 7.5 to 8.0 was reached, after which the urine was then re-acidified to a pH of 5.5. This was done to dissolve the conjugated pyridium elimination product which sometimes precipitates at these pH values on standing for twenty-four hours and also to dissolve any uric acid which might have been precipitated.

The pH 5.5 was selected because this is near the pH at which urine is voided in normal persons and for the reasons given in the following discussion. At this pH the calcium phosphates, magnesium ammonium phosphates, calcium carbonates, and most of the ammonium urates are dissolved. Thus almost all the salts on the alkaline side which might occupy a very large volume and give false information to the determination of the "organized" sediment will be completely avoided. The only salt that will not dissolve readily at this pH is calcium oxalate, and while it is present in many cases, it contributes a negligible amount to the volume of the precipitate. A pronounced case of oxaluria was not seen during the course of these studies, even though the normal cases were medical students who were under great stress and anxiety due to scholastic examinations.

On the acid side, between pH 5.5 and pH 7, there is a danger of some sodium urate[2] precipitation, but the patients usually have an output of about 2,000 c.c., and in twenty-four hours, at room temperature, very little of the sodium urate will precipitate unless the patient has a fever.

The determinations of the sediment were run in Goetz phosphorous centrifuge bottles with a volume of 100 c.c. and a tip of 1 c.c. capacity, and calibrated in 0.05 c.c. graduations from which estimations of ±0.01 c.c. could be made. The specimens adjusted to a pH of 5.5, as indicated above, were centrifuged for fifteen minutes at the rate of 1,800 ±300 r.p.m. At this speed, for the given time, variations in sediment volume are negligible for a few minutes before or after the fifteen minutes' interval, so that it can be concluded that the packing of the sediment has reached a relatively constant point. The nebecula, which consists of mucin, is insoluble at this pH. It descends in the centrifuge bottle as a light voluminous opalescent flocculation and is not to be considered as a part of the organized sediment. The separation between the nebecula and the solid opaque organized sediment is usually sharp, so that no difficulty in reading the sediment is encountered. In the case that the separation is not distinct, an error in reading of about ±0.03 instead of the 0.01 is given, and the best estimation possible is made. This type of difficulty does not occur very often.

It is granted here that in a twenty-four-hour specimen, especially in alkaline solution, cytolysis tends to decrease the sediment volume, but a compensating factor of protein swelling tends to keep this error down. Since the sediment in succeeding twenty-four-hour samples of the same patient is compared, this error will be relatively constant over a short period.



Graph 1.—Distribution of 40 cases of urinary infections with regard to organized urinary sediment variation per 100 c.c. of urine following pyridium therapy for approximately two weeks.

While this work was undertaken principally for the purpose of sediment study, the symptoms of burning and nocturia were carefully noted. In almost all cases in which these symptoms were reported, there was a marked decrease in nocturia and in the severity of the burning following the administration of pyridium. Some cases were spectacular with respect to the rate at which a severe burning would disappear, giving evidence of the reported analgesic power of pyridium. In some of these cases a severe burning would be completely gone after twenty-four to forty-eight hours' use of pyridium. Results such as these are a confirmation of all the clinical studies made by previous investigators. This part of the work was done in an effort to correlate the reduction and disappearance of the symptoms with a decrease in the sediment.

In Table I no attempt was made to classify the types of cystitis. Not all patients were cystoscoped. The diagnoses of those patients who were not cystoscoped were made on the basis of symptomatology and demonstrable pus cells in a fresh urine specimen. Undoubtedly there were a few cases of pyelocystitis in this group. The symptoms of burning, dysuria, and nocturia are listed opposite the percentage of reduction to facilitate comparison of the symptomatology changes with the reduction in sedimentation per 100 c.c. Where

TABLE I*

CYSTITIS	PRELIMINARY SED./100 C.C.	SED./100 C.C. WITH PYRIDIUM 2 WEEKS	AMOUNT OF REDUCTION SED./100 C.C.	% OF REDUCTION SED./100 C.C.	BURNING OR DYSURIA	NOCTURIA
1 M	0.78	0.13	0.65	83.3	--	---
2 M	0.55	0.17	0.38	69.1	--	---
3 M	0.65	0.1	0.55	84.6	--	---
4 M	0.30	0.03	0.27	90.0	--	---
5 M	0.24	0.07	0.17	70.8	--	---
6 M	0.43	0.6	0.17 inc.	39.2 inc.	--	---
7 M	1.30	0.1	1.20	92.3	--	---
8 M	2.3	0.15	2.15	93.5	--	---
9 F	0.20	0.25	0.05 inc.	25.0 inc.	--	---
10 M	0.15	0.30	0.15 inc.	100.0 inc.	--	---
11 M	0.63	0.15	0.48	76.2	--	---
12 M	0.30	0.15	0.15	50.0	Less	Less
13 M	0.25	0.24	0.01	4.0	Less	Less
14 M	4.0	1.1	2.9	72.5	--	---
15 M	2.0	0.18	1.82	91.0	--	---
16 M	1.8	0.43	1.37	76.1	Less	Less
17 M	2.0	0.45	1.55	77.5	Less	From 20× to 2×
18 M	0.20	0.35	0.15 inc.	75.0 inc.	--	---
19 M	0.57	0.05	0.52	91.2	Gone	From 5× to 1×
20 M	1.0	0.20	0.80	80.0	Less	From 3× to 1×
21 F	0.32	0.03	0.29	90.6	Gone	From 4× to 1×
22 M	0.45	0.26	0.19	48.0	Less	From 10× to 4×
23 M	0.34	0.25	0.09	26.5	Less	Same
24 M	0.45	0.21	0.24	53.3	--	---
25 M	0.25	0.03	0.22	88.0	Gone	Same
26 F	0.25	0.20	0.05	20.0	Gone	---
27 M	0.48	0.24	0.24	50.0	Gone	None
28 M	0.34	0.09	0.25	73.6	--	---
29 M	0.15	0.02	0.13	86.7	Less	Less
30 M	0.70	0.10	0.60	85.7	Gone	From 15× to 8×
31 M	0.25	0.13	0.12	48.0	Same	Same
32 M	0.70	0.18	0.52	74.3	Less	Less
33 M	0.12	0.03	0.09	75.0	Less	From 15× to 8×
34 M	0.14	0.02	0.12	85.7	None	None
35 M	0.65	0.02	0.63	97.0	Less	None
36 M	0.65	0.45	0.20	30.4	--	---
PYELONEPHRITIS						
1 M	1.1	0.13	0.97	88.2	--	---
2 F	0.40	0.06	0.34	85.0	Less	Less
3 M	0.26	0.06	0.20	77.0	Less	Less
RENAL INFECTION FOLLOWING NEPHROLITHOTOMY						
1 M	2.00	0.30	1.70	85.0	Less	Less
NORMAL CASES						
1 M	None	None	0	---	No symptomatology	
2 M	0.02	None	0.02	---		
3 M	None	None	0	---		
4 M	None	None	0	---		
5 M	None	None	0	---		

*The percentage of reduction of the total sediment would give a result similar to Table I. Because of errors in volume, inherent in the collection of twenty-four-hour specimens from hospital and ambulant patients, The author feels that the expression of the results in sedimentation per 100 c.c. is more justified and better controlled than the total sediment.

M = male.

F = female.

inc. = increase.

there is a line through the space or spaces in the symptomatology columns, no information was available. *Gone* means that symptoms were present and have completely disappeared. *Less* means symptoms were present and the severity has decreased considerably. *Same* means that symptoms were present and that they have not improved. *None* means that symptoms were not present. In no case was the severity of the symptoms increased following pyridium administration. Other symptoms recorded were not put on the chart due to space limitations. Wherever the expression "sed. per 100 c.c." occurs, it will be understood to mean cubic centimeters of sediment per 100 c.c. of urine.

COMMENTS

Table I and Graph 1 show strikingly the effect of pyridium on the reduction in the amount of organized sediment in cases of urinary infections. Eighty per cent of these cases show a decrease in sediment of 50 per cent or more. In those cases in which observation of burning and dysuria were recorded, 95 per cent showed a decrease in the severity or a disappearance of these symptoms. As can be seen from the chart, a good correlation exists between the reduction of the sediment and a decrease in the severity of the symptoms. In a few cases an improvement of the symptomatology took place without a corresponding decrease in the sediment. Normal persons do not have any appreciable organized sediment before or after pyridium.

SUMMARY

1. A technique has been presented for the determination of the organized urinary sediment volume.

2. Normal persons have a very low organized sediment before and after pyridium administration, between 0.0 and 0.02 c.c. per 100 c.c.

3. Pyridium is very effective in reducing the organized sediment in cases of urinary infection, especially cystitis and pyelonephritis.

4. With very few exceptions the reduction of the organized sediment is accompanied by a decrease or disappearance of the subjective symptoms.

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THE MODE OF ACTION OF BRAN*

I. Effect of Bran Upon Composition of Stools

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INTRODUCTION

THE extensive use of bran as a laxative and in diets justifies an inquiry into its various properties. This report deals mainly with the physical and chemical qualities of bran stools. It is aimed to discover the mode of action of bran and to elucidate, as far as possible, the effect the alimentary tract has upon bran and the effect bran has upon the alimentary tract.

METHOD OF STUDY

These investigations were carried out on approximately 200 healthy persons, for the most part male medical students between 20 and 30 years of age. A few healthy women subjects were also used.

To make the studies of practical value these persons were placed on uncontrolled and unrestricted diets, just as are the public consumers of bran. The individuals collected their stools in specially prepared asphalt paper bags† for three to six days (prebran period). This control period was followed by a second period, which varied in different experiments from three to fourteen days, during which time one ounce of bran (Kellogg's "All-Bran") was added to the daily diet (bran period). A third period of several days followed (after-bran period). All persons had normal bowel habits; stools were obtained regularly every day with very few exceptions.

At present the only consistent criterion of the laxative action of bran is the change in stool weight. Whenever this has been definitely increased, we speak of laxative action. When the weight is decreased or not changed, it is spoken of as lack of such action. It should be admitted that this criterion is not the only one. Bulk is perhaps more important, but it has not been possible to measure it accurately. The consistency of the stool may be still more important for it may possibly be more constantly affected by bran, and "penetrometric" studies to investigate this matter are reported. The frequency of the stools is important, but it was not possible to secure a record that gave significant results in these normal persons of fixed habits. The rapidity with which the bran stool marked with barium passes through the alimentary tract has been carefully

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studied,[1] but it does not seem to give better results than those based upon the weight, and a comparison of the results using weight as the criterion with results using speed as the criterion will be discussed in a subsequent report.

Bran contains approximately 8 per cent of crude fiber. Thus the usual one ounce serving of bran supplies only 2.4 Gm. of this material. This crude fiber comprises about 2 per cent of the total dry stool weight. Obviously, the bulk-forming properties of bran are not due to this small quantity of crude fiber per se.

The hydrophilic properties of bran crude fiber are negligible. Therefore, water absorption by bran crude fiber is likewise eliminated as an important factor in the formation of bulky stools.

A change in stool consistency almost always appears after the ingestion of bran: bran stools are generally softer. The change in consistency could not be accounted for by changes in moisture content, since this varied little from the control stools.

To determine moisture, about 2 Gm. of well-mixed stool were weighed on a watch glass and dried in an oven at 100° C., cooled, and weighed again; the drying and weighing were continued until the weight was constant.

In one series 7 persons (18.4 per cent) showed no change in the moisture content of bran stools. Eight persons (21 per cent) showed a decrease in moisture content, the decrease being slight (average 4.5 per cent, maximum 6 per cent). Twenty-three persons (60.6 per cent) showed an increase in moisture to a very slight degree (average 4.9 per cent, maximum 17 per cent). Bran stools definitely appeared soft, but it is impossible to distinguish between 5 per cent increase or decrease in water content by the appearance alone. While moisture may play a part in the increased softness of bran stools, the increase is not sufficiently constant to account for any substantial change in consistency. The softness is, no doubt, also due to gas. To determine whether softness is a constant change in all bran stools and to express the change quantitatively, penetrometric studies were undertaken.

PENETROMETER STUDY

The penetrometer is intended to measure the consistency of homogeneous materials. Stools are not homogeneous and, in addition, the quantity of gas which is present before expulsion of the stool from the gastrointestinal tract is no longer present. Gas is lost in transferring the specimen from the original container to the measuring apparatus and during the testing. Thus far the results of the penetrometric studies have been entirely unsatisfactory.

SPECIFIC GRAVITY OF STOOLS

Attempts to measure accurately the gas content of stools have thus far proved unsatisfactory. A considerable portion of gas is lost at defecation and during transportation, storage, and the handling of stool specimens.

Bran stools, probably due to gas content, have a lower specific gravity than control stools. It is, therefore, probable that the increase in bulk is actually greater than the increase in weight, in some cases to a considerable extent.

However, an attempt was made to develop a method for measuring the specific gravity of stools. None of the methods recommended in the literature proved satisfactory for the purpose. A liquid was required that would not dissolve or penetrate into the stool; therefore, water and similar fluids were excluded. After searching for a nonaqueous liquid and one that would not evaporate too rapidly, a petrolatum-benzin (10 per cent benzin) mixture was chosen. Approximately 5 Gm. of feces were placed in a weighed graduated centrifuge tube carefully weighed again and the petrolatum-benzin mixture was added from a burette up to the 15 c.c. mark. The weight of the specimen divided by the difference between 15 and the number of cubic centimeters of liquid added gives the specific gravity of stool.

A comparison of weight and volume in the bran and afterbran periods showed that in some cases the difference in bulk is much more obvious than that in weight. This method was abandoned because of difficulties encountered in its use.

CHEMICAL EXAMINATIONS

The pH of the stools, the total fat content, the amount of volatile fatty acids, and the relative proportion of butyric to acetic acid were determined.

Olmsted[2] stated that the laxative effect of bran is produced in large part by the volatile fatty acids originating from the breakdown of bran by bacteria.

Hydrogen-ion determinations (pH) were made using a quinhydrone electrode potentiometer. Solid stools were diluted two to three times with saline. In one series, the stools of 36 persons (65.4 per cent) showed a slight decrease in hydrogen-ion concentration, and those of 19 (34.6 per cent) showed a slight increase during the bran period.

The stool fat was determined by adding a quantity of 1 per cent alcoholic hydrochloric acid solution equivalent to five times the weight of the sample. This mixture was stirred well and dried on a water bath. The product obtained was extracted in a Soxhlet for twelve hours with ether. This procedure gives the total amount of nonvolatile fatty acids. During the bran period 18 per cent of 67 persons showed an increase in stool fat content, and 82 per cent showed a decrease.

Kjeldahl nitrogen determinations were made on a known quantity of dried stool. The nitrogen content of the stools was examined in the prebran, bran, and afterbran periods of some of the individuals who were on controlled diets. An increase in nitrogen was found during the bran period, probably due to a more rapid passage of the stools through the gut. The increase in nitrogen was not very marked and soon returned to normal in the afterbran period.

The volatile fatty acids in the stools were determined by releasing them from their salts by the addition of a stronger acid. About 150 c.c. of 10 per cent oxalic acid and water were added to approximately 10 Gm. of fresh stool (accurately measured) in a flask which was connected to a condenser system. The acids which passed over with the steam were taken up in a known quantity of N/5 sodium hydroxide. After the distillation was finished, the solution was titrated with N/5 sulfuric acid. The difference between the amount of alkali

in the receiving flask before distillation and the amount after distillation as indicated by the titration with N/5 sulfuric acid, gives the quantity of volatile fatty acids.

These volatile fatty acids are found to be composed chiefly of acetic and butyric acids. However, there is no correlation between an increase in stool weight and the relative proportion of acetic to butyric acid.

The amount of volatile fatty acids varied greatly in all the experiments. No uniform relationship between butyric and acetic acid was noted. Volatile fatty acids are present in the gut combined with cations, such as potassium and calcium. It must be considered doubtful whether the salts of these acids contribute to the laxative action of bran. A very careful statistical study of the question showed that the correlation between volatile fatty acids and laxative action (as measured by increased stool weight) is so slight (0.09) that it cannot be considered significant from the statistical standpoint.

CONCLUSIONS

1. The most significant change in stools following the addition of bran to an uncontrolled diet is a softening and an increase in stool weight.

2. Bran stools show an increase in bulk greater than the increase in weight. There is little variation in the percentage of water.

3. There is an increase in volatile fatty acids, chiefly acetic and butyric acids, but this increase is not proportional to the increase in weight.

4. The proportion of acetic to butyric acid showed no consistent correlation with laxative action as measured by an increase in stool weight.

We are grateful to Miss Elizabeth M. Adles for assistance in statistical studies.
Protocols for all experiments are available.

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LABORATORY METHODS

GENERAL

THE PRODUCTION OF ARTIFICIAL TUMORS IN THE BRAINS OF CATS*

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THE method used in these experiments for the production of so-called artificial tumors in the brains of animals is not a new one. Rather, it is a modification of the method employed by van Schultén,[1] Hill,[2] and later in 1909 by Cushing and Bordley.[3] Van Schultén screwed small tubes into the skulls of animals for the purpose of injecting substances into the subarachnoid space and for recording cerebrospinal fluid pressures. Hill's study of acute cerebral compression was based in part on a similar technique. His historical review of the subject is especially interesting. Cushing and Bordley used the same method in their studies, demonstrating the mechanical relationship that exists between increasing intracranial pressure and retinal edema. In some of their experiments paraffin was injected, and they noted no permanent "effect beyond that occasioned by immediate local destruction of tissue." A search of the literature has revealed no further application of this method as we have used it.

These experiments demonstrate a method whereby a foreign body, which can be increased gradually in size over a period of weeks or months, may be introduced into the brain of an unanesthetized animal and controlled in its growth. Further, they indicate some of the effects of such foreign bodies. Van Schultén's tube has been modified to resemble a grease cup, and the cups have been filled with sterile beeswax softened to a proper consistency with lipiodol. The mixture was injected slowly by turning the cup down a little each day, and has been found of itself to be relatively inert for the periods of time employed (Fig. 3). Only slight inflammation was observed in the walls around the wax.

For each experiment, under aseptic conditions, a grease cup plug was screwed into a trephined hole in the skull of an animal. In all 34 cats were used. The dura under the hole was cut away before the plug was implanted and afterwards the skin was closed around it. Tubes were placed in the skulls over most of the accessible parts of the cerebral hemispheres. Records were made of the general health of the animals, and the placing reactions, described by Bard and Brooks,[4] were tested at about daily intervals, both before and after the cups

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were turned down. Observations on the optic disks, and x-ray pictures of the skulls of some of the animals were made at longer intervals. Meningitis and brain abscesses developed in several of the animals and these were discarded. With the appearance of pressure symptoms, the cats were anesthetized with nembutal, and the vascular system was injected with 10 per cent formalin. The brains were then removed for gross, and in some instances microscopic, examination.

RESULTS

It was found that the shape of the tumors could be controlled within limits by varying the relative concentration of wax and lipiodol. Mushroom-shaped masses lying under the dura were produced by the use of a mixture predominantly lipiodol (ratio 5:1). Long cylindrical masses were formed when the mixture was chiefly beeswax (Fig. 1). Spherical tumors lying below the surface of the brain were formed when a mixture of two parts of lipiodol and one of wax was used (Figs. 2 and 4).

Histologic examination of the tumors and surrounding brain tissue in sections stained with hematoxylin and eosin showed that the foreign body produced its direct effect by compression, displacement, and distortion of the normal architecture of the brain, and indirectly in some instances by blockage of the cerebrospinal fluid passages so that hydrocephalus developed. A complete fibrous capsule containing capillaries, and continuous with the dura was found around most of the tumors (Fig. 3). The pia-arachnoid appeared to be fused with this capsule at the surface of the brain where the greatest proliferation of fibrous tissue was present. A small number of leucocytes was found in the capsule, particularly on its inner surface, and some fibrin. No changes in the character or number of the supporting cells of the brain tissue itself were observed with the hematoxylin and eosin stains.

A modified Weigert's stain was applied to sections obtained from the brain of Cat 5. The rounded tumor (Fig. 2) was located just back of the motor area under the lateral gyrus on the right; it had penetrated 2 cm. downward into the brain, pushing the corpus callosum downward about 8 mm. Sections through the brain about the foreign body showed, as might be expected, a marked decreased staining capacity of the myelin sheaths of the fibers in the corpus callosum immediately under the mass. Thirty-seven days elapsed during the production of the mass, and approximately 1 c.c. of the wax mixture was injected.

In these experiments the first effect of the increasing size of the foreign body was a disturbance of the normal placing reactions. This was true no matter where the grease cups were located. However, different placing reactions in different legs were primarily affected, depending on the location of the masses. The order of appearance of deficiencies in the placing reactions resulting from wax tumors placed on different areas appears to offer some evidence as to the localization of these reactions in the cortex. On the other hand, for proper interpretation, it must be considered that transmitted pressure can be an important factor in the production of these deficiencies.

The accumulation of a mass in the middle portion of the anterior sigmoid gyrus, a region known to be a contralateral foreleg area primarily, produced first a deficiency in the abduction response in the contralateral foreleg. In another animal, pressure of the foreign body on the posterior sigmoid gyrus led to a deficiency in the contact response in the contralateral forelegs and hindlegs first. This evidence of localization is to be expected, since this region, which has been shown to be the cortical area of motor representation for these extremities (Ward and Clark[5]), has also been shown by strychninization (Dusser de Barenne[6]) to be a part of the area for sensory representation of the contralateral foreleg and to a less extent, the contralateral hindleg.

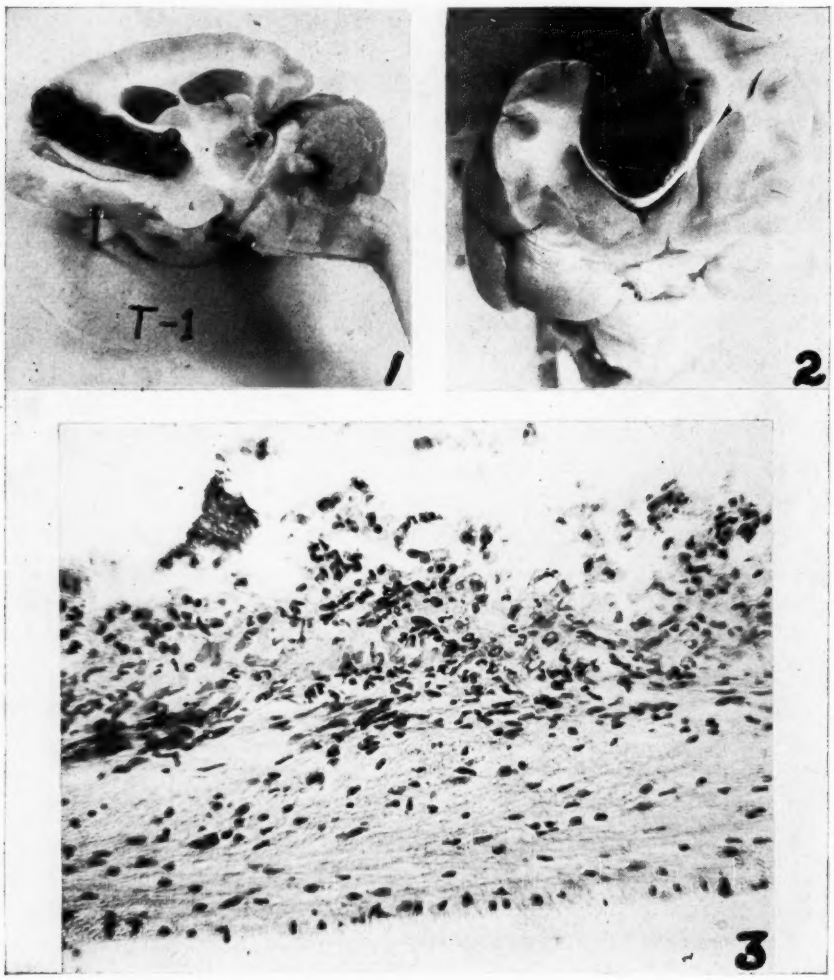

Fig. 1.—Cylindrical foreign body formed over a period of sixty-nine days (cup turned down 14 times, one turn each time). Marked hydrocephalus developed, and herniation of the cerebrum (tentorial surface) under the bony tentorium is seen at arrow.

Fig. 2.—Spherical foreign body formed over period of thirty-five days; 20 (turns) increments of wax were added. A degeneration of the myelin sheaths was noted with Weigert's stain in the corpus callosum.

Fig. 3.—Section through wall around the foreign body and the corpus callosum from cat's brain pictured in Fig. 2. Ependymal lining of lateral ventricle seen below. (See text. H. & E. Oil ×215.)

In contrast to the relatively immediate deficiencies that occurred when the foreign bodies were placed in the anterior one-third of the brain, an increasing delay in the appearance of symptoms was found when the masses were more and more posteriorly placed; that is, more increments of wax (from 3 to 10 turns of the grease cup) were required before the appearance of placing reaction deficiency.

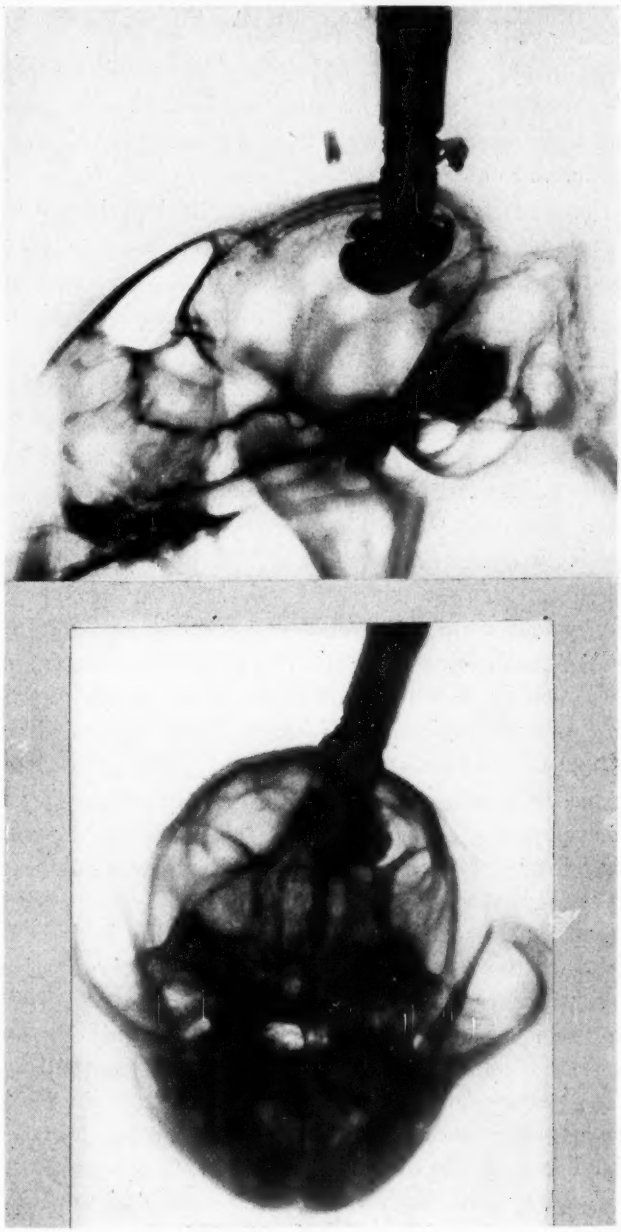

Fig. 4.—X-ray pictures of spherical foreign body formed in nineteen days (15 turns).

In 8 cats with masses on the posterior two-thirds of the cerebrum, the first alteration of the normal activity was noted to be a slowing in the contact reaction in the contralateral hindleg. Bard and Brooks have shown the integrity of the placing reactions to be dependent on the area immediately around the cruciate sulcus and the most anterior end of the lateral gyrus. The results of experiments in which the masses were located in various parts of the posterior two-thirds of the cerebrum demonstrate the effect of the pressure exerted either on a "center," or on nerve pathways leading to or from such a "center." It does not appear likely that the cortical "centers" subserving the placing reactions are those affected by the pressure of these remote masses, because when pressure is exerted directly against these regions, either different placing reactions or combinations of placing reactions are affected first. A selective effect of pressure transmitted downward to the centrifugal or centripetal cortical pathways appears to offer a more likely explanation. Whether this is due to a selective loss of function like that which occurs with pressure on a peripheral nerve (Gasser and Erlanger[7]), or whether it is the result of pressure on the blood vessels supplying these regions is not known.

Remissions of symptoms frequently occurred when more than twenty-four hours elapsed between successive additions of wax. This is illustrated in an animal (Cat 19) with the grease cup on about the middle of the right lateral gyrus close to the midline. The cup was turned down one turn each day for the first seven days. The contact placing reactions after the first forty-eight hours became progressively slower and smaller in both hindlegs. The responses of the forelegs remained normal. No increment was added on the eighth day, and within the next twenty-four hours all reactions became normal. The contact response in the hindlegs again was slightly reduced on the eleventh day after the additions of wax were resumed on the ninth and tenth days. X-ray pictures of the skull showed that the mass occupied a position on both sides of the midline in the posterior one-third of the cerebral cranial cavity (Fig. 4).

The location of the masses in the cranial cavity appeared definitely related to the time of appearance and intensity of the symptoms of compression. The slowly enlarging foreign bodies produced displacement of the brain tissue, and in some instances this displacement was enhanced by the additional factor of hydrocephalus, the hydrocephalus being caused by the indirect blockage of the drainage passages of the cerebrospinal fluid by the foreign bodies. Autopsy revealed the presence of pressure cones in the animals that survived for several weeks or more, though dilated ventricles were present only in some of these. In those with undilated ventricles the tumors were frequently large and usually situated so as to produce most of their displacement from the front of the cranial cavity. When situated more posteriorly, tumors formed by slower and less frequently added increments of wax tended not to be associated with dilated ventricles, even though tumors similarly situated did lead to distended ventricles if they were formed more rapidly. In all probability the factor of rate of growth accounts for the lack of early symptoms in certain human cases in which enormous, slow-growing tumors have eventually manifest themselves.

As the wax masses were increased in size, the effects on the animals presented a fairly constant pattern. A blurring of the edges of the optic disks with engorgement of the veins was seen. The cats sat very quietly, back humped, nose down, neck extended, feet bunched. When they were handled, they were found to be tense and at times cried angrily and lashed their tails. They were usually unsteady on their feet, and the placing and hopping reactions were lost in all legs. They appeared to prefer to lie on the side opposite the mass if the mass was definitely asymmetrical in its position. The pupils were usually dilated and responded to light slightly, or not at all; and vision appeared to be impaired in that the animals often ran into objects as they moved about.

Terminally the animals exhibited some of the symptoms characteristic of decerebrate rigidity. They were no longer able to stand, and their heads and necks were thrown back. While the cats were lying undisturbed, their forelegs were extended and retracted, and their hindlegs were partially flexed. The Stutz reaction was easily elicited in the forelegs. Local reflex activity was unimpaired. Slow running movements occurred at times in the course of this stage. Death followed shortly. For example, in Cat 34 the mass was located close to the midline and in the middle third of the cerebrum and had been produced rapidly (16 turns, 2 each day). Death occurred on the eighth day, though no symptoms appeared until the seventh day. Autopsy showed slightly dilated ventricles, a cerebral herniation at the tentorium and a cerebellar pressure cone at the foramen magnum. A bony tentorium cerebelli in the cat is probably responsible for some of the symptoms of decerebration that are to be observed in these acute stages. Compression of the brain stem under the tentorium occurs with the herniation of the pulvinar and the tentorial surface of the cerebrum. This herniation was observed in all animals allowed to go to this advanced stage (Fig. 1).

SUMMARY AND CONCLUSIONS

The experiments demonstrate a method whereby foreign bodies that simulate growing benign tumors can be placed on various parts of the brains of animals.

The masses give rise to localizing symptoms, and a chronic state with evidence of increased intracranial pressure can be produced.

An acute state of increased intracranial pressure may develop with rapid injection of the wax mixture; hydrocephalus may develop and become a factor in the production of the symptoms. Some symptoms of decerebration characterize this state.

The masses can be visualized in the living animal by x-ray pictures.

The experiments indicate in another way that parts of the motor area in the cat subserve certain placing reactions in different extremities. Of these placing reactions it was found that the contact reaction was the most labile in that pressure exerted on parts of the cerebrum other than the motor region caused an elimination of this reaction before abolishing the others.

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A METHOD FOR PREPARING SMEARS AND SECTIONS OF ASPIRATED STERNAL MARROW*

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THE diagnostic value of biopsies of sternal marrow is so firmly established that the time may soon arrive when failure to examine the marrow of a patient with an obscure hematologic disorder will have medicolegal significance. Many instruments have been devised, and several methods have been suggested to facilitate the procedure of sternal biopsy. These fall into two general categories: aspiration and trepanation techniques. The latter possesses a number of advantages as pointed out by Custer,[1-3] Dameshek, Henstell, and Valentine,[4] and Hynes.[5] It also has important limitations, as emphasized by Young and Osgood.[6] Some workers have devised instruments by means of which solid fragments of spongy bone may be removed from the sternum by a modified trephine technique.[7, 8] However, all the various trepanation methods have the serious disadvantage of being relatively formidable procedures which cannot be repeated at short intervals. The advantages of the aspiration technique are so many that it is the method of choice of most clinicians. It has one serious drawback—the lack of organized tissue makes impossible a well-oriented study of tissue relationships. In order to overcome this difficulty, it has been suggested[9-11] that smears may be made of some of the marrow and the remainder be allowed to clot. The clot may then be fixed, embedded in celloidin or paraffin, sectioned and stained. Reich,[12] and Vogel, Erf, and Rosenthal[13] concentrated the marrow by centrifuging it and made smears from the buffy coat. The method described below is a further modification of the foregoing and permits the making of smears from sternal marrow both before and after concentration by centrifuging, as well as the preparation of tissue sections. It requires no special instruments, avoids excessive hemodilution, and yields material which is adequate for diagnosis in almost every instance.

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METHOD

An 18 gauge spinal puncture needle is filed through 1½ inches from its shaft, its point is rebeveled, and a fine mark, 0.6 cm. from the tip, is made on it with a file. The purpose of this mark is to provide a convenient guide whereby the operator may judge the depth of penetration of the needle when in use. The usual thickness of the outer table of the sternum is 0.4 cm.; hence, when the file mark is flush with the skin, the point of the needle is usually within the marrow cavity. The puncture site, preferably in the midline between the second and third ribs, is cleansed with iodine and alcohol and infiltrated with novocain solution. The novocain should be injected into the skin, subcutaneous tissues, and periosteum. The puncture needle, with stilet in place, is pushed through the soft tissues and into the outer table of the sternum. The operator should hold the needle in such a way that the file mark is grasped firmly between his thumb and index finger, while the broad base of the needle fits snugly into the palm of his hand. This enables him to push the needle into the marrow cavity without losing control of the instrument.

Penetration of the cortex of the bone is facilitated by rotating the needle slowly as it is pushed in with a steady pressure. When the file mark is flush with the skin, the stilet is withdrawn, and a 5 or 10 c.c. syringe is fitted to the needle. Trial aspiration is made by applying negative pressure to the syringe. Usually marrow mixed with blood is obtained at the first trial. Sometimes it is necessary to remove the syringe, replace the stilet, and go a little deeper in order to obtain marrow. This happens especially if the patient is obese, or if the skin is edematous. Experience soon permits the operator to gauge the thickness of the subcutaneous panniculus while he is infiltrating the tissues with novocain. If, in his judgment, the soft tissues are unusually abundant, he may hold his thumb and index finger "stop" a corresponding distance above the file mark, which is based upon the average thickness of the soft tissues and the outer table of the sternum. The sternal marrow should be withdrawn rapidly, using strong negative pressure. This occasions a moderate feeling of discomfort but aids in dislodging minute plugs of marrow. It is not advisable to withdraw more than 1.0 c.c. of marrow in order to minimize hemodilution. As soon as the syringe is filled to the 1.0 c.c. mark, the needle and syringe should be withdrawn, and their contents emptied into a small test tube containing sufficient dry oxalate to prevent clotting. Pressure is then applied to the sternal puncture site and a sterile dressing is affixed.

Smears are made immediately from the "oxalated" marrow, dried in air, and stained with Wright's stain. The undiluted stain is allowed to act for two minutes, care being taken to avoid evaporation and precipitation of the stain. If some of the stain becomes precipitated, an additional drop or two will dissolve the precipitate. The stain is diluted with distilled water and allowed to remain on the slide for six to eight minutes longer. The stain is washed off with distilled water and the smears may be examined immediately, using the low-power objective to ascertain whether or not they are satisfactory for diagnostic purposes. If the smears are considered satisfactory, the remainder of the marrow is centrifuged at low speed for fifteen to twenty minutes. The

clear supernatant fluid is pipetted off, and additional smears are made from the concentrated buffy layer. These are stained with Wright's stain as previously. The remainder of the marrow is removed gently from the tube and placed on a small piece of dry filter paper, to which it will adhere firmly. The filter paper with adherent marrow is placed in 10 per cent aqueous solution of formalin, dehydrated in alcohol, cleared in xylol, embedded in paraffin, and sectioned. The sections should be from 4 to 6 microns thick, and several duplicate sections

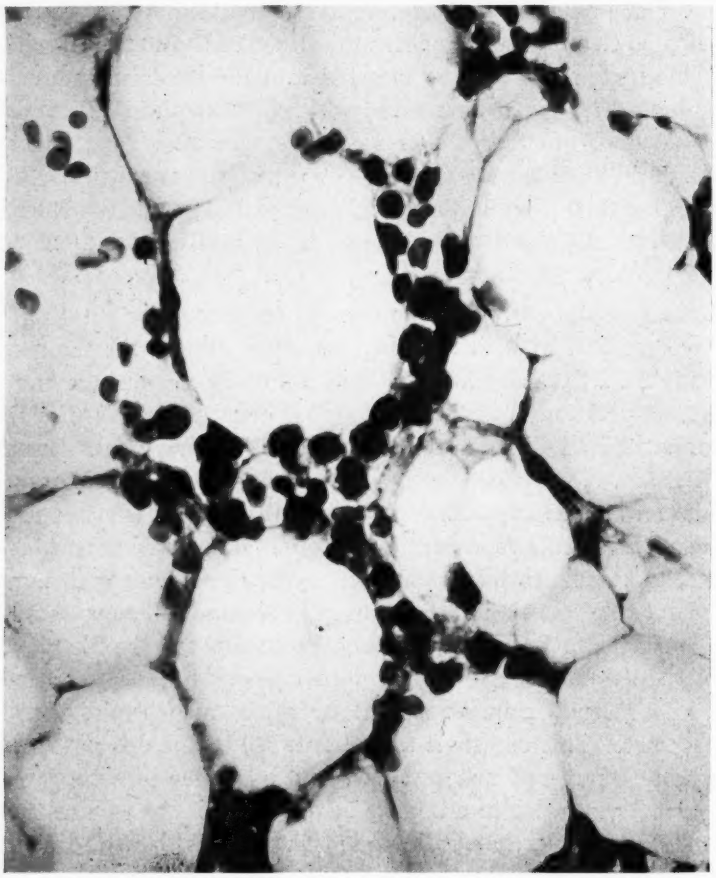

Fig. 1.—A fragment of aspirated sternal marrow. Hematoxylin and eosin stain. Magnification 190 diameters (approximately). The marrow was removed from a white male, aged 22 years. Although the marrow was hypoplastic (confirmed at necropsy), two small fragments of organized marrow tissue were obtained. Both showed a marked granulocytopenia, a moderate decrease of erythropoiesis, and a diffuse fatty replacement of the marrow.

may be mounted on each of two slides. They may be stained with hematoxylin and eosin or with methylene blue, eosin-phloxin.[14] Small fragments of marrow will usually be present in the sections. These frequently appear as well-organized pieces of tissue, even in a marrow which is hypoplastic (Fig. 1). In leucemic marrow, groups of atypical cells often are collected in nests about small vessels (Fig. 2). Occasionally no organized tissue is found in the sections, and sometimes the centrifuged material disintegrates as it is sectioned. However, during the three-year period the method has been in use, very few such failures have been encountered. Moreover, in the occasional case in which no tissue frag-

ments are found, the smears still are available for examination. One other point is worthy of emphasis: in no instance has sternal puncture caused local implantation metastases in my experience and no instance of such a sequel can be found in the available literature. This is of considerable practical importance and is very remarkable in view of the fact that thousands of sternal punctures have been performed during the past few years. It is all the more significant when it is considered that many of these punctures revealed the presence of a variety of malignant neoplasms in the sternal marrow.

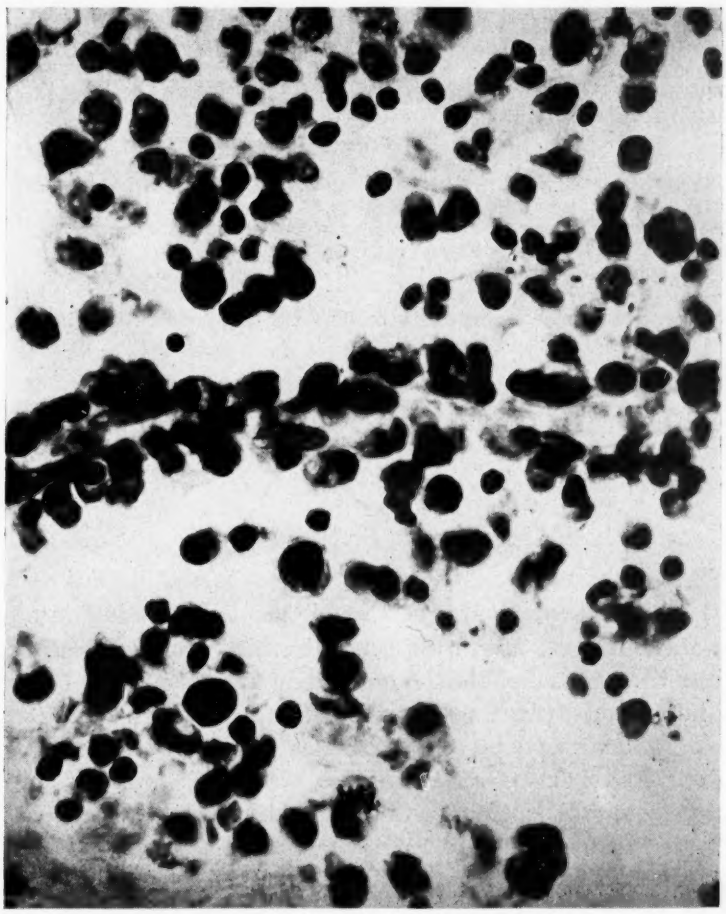

Fig. 2.—Sternal marrow obtained by aspiration. Hematoxylin and eosin stain. Magnification 190 diameters (approximately). The marrow was aspirated from a patient with diffuse plasma cell myelosis. Note the small vessel in the center and the groups of plasma cells in the adjacent perivascular spaces.

SUMMARY

A method is described whereby sternal marrow may be obtained by aspiration and prepared in the form of both smears and tissue sections.

Attention is directed to the fact that no examples of implantation metastases have been encountered following aspiration of sternal marrow.

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ISOPROPYL ALCOHOL AS A DEHYDRATING AGENT AND PRESERVATIVE FOR BIOLOGICAL SPECIMENS*

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DURING recent years isopropyl alcohol has been coming into wider use as an antiseptic and sterilizing agent, but little emphasis has been placed on its applicability to general biological routine processes in the laboratory. Its use in the latter connection was first pointed out by Griffin.[1] Isopropyl alcohol, C_3H_7OH, is an inexpensive by-product of the oil industry, with properties very similar to ethyl alcohol. It costs less than tax-free 95 per cent ethyl alcohol. It is not palatable as a beverage and is not subject to the extensive bonds and responsibilities required in the use of tax-free ethyl alcohol. It is a water clear liquid, completely miscible with distilled water. It is sold commercially as anhydrous isopropyl alcohol, with less than 1 per cent of water and other impurities, and as such acts as well as absolute ethyl alcohol in dehydrating procedures. In the preparation of histologic and pathologic material, it is the equal of ethyl alcohol as a dehydrating agent, preservative, and solvent for stains. We have used it extensively in the staining procedure for the diagnosis of parasitic protozoa in fecal smears with excellent results. In fact, we have entirely replaced it for ethyl alcohol for all purposes in our laboratory.

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A PRACTICAL METHOD FOR INTRASTATE EVALUATION OF THE PERFORMANCE OF SEROLOGIC TESTS FOR SYPHILIS*

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INTRODUCTION

THE improvement of laboratory tests used as aids in the diagnosis of disease is a matter of fundamental importance, not only to the medical profession but also to the public as well. Refinements in technique and new discoveries have added much in accuracy in recent years to many laboratory tests; however, constant vigilance must be exercised to insure that new procedures do not sacrifice too much in specificity in the effort to attain speed and sensitivity.

In those states where the State Department of Health maintains a Bureau of Laboratories, consisting of a central laboratory and several branch laboratories, and in those states where private clinical laboratories are officially authorized to perform certain kinds of examinations, under supervision of the State Board of Health, some means of checking performance is essential. This is true particularly where laws are in effect requiring certification of freedom from venereal disease, based on physical examination and certain laboratory tests, before marriage licenses may be obtained. Usually these tests consist of serologic tests for syphilis, and, where indicated, microscopic examinations of smears for evidence of gonorrhea. It is true, of course, that while the results of these laboratory tests per se are not sufficient grounds for denial of a license, the actual tests performed should, nevertheless, be of the highest degree of reliability, i.e., they should be above question as to their sensitivity and specificity.

A survey of the literature relating to the serodiagnosis of syphilis is likely to leave one confused due to the multiplicity of tests from which to choose. It should be remembered, however, that in the last analysis the value of any technique depends, first, on its specificity, that is, its failure to give positive reactions in conditions other than syphilis; and second, on its sensitivity, that is, the proportion of actually syphilitic patients detected by the test. The very multiplicity of available procedures, and the conflicting claims made for them, is sufficient indication that the perfect test has not yet been devised. The most practical way of evaluating this or that particular technique is, therefore, by properly controlled comparisons on parallel series of specimens.

SEROLOGIC CONFERENCES

Within recent years several official attempts have been made to evaluate the merits of various complement fixation and flocculation procedures. In the first place conferences were conducted by the health organization of the League of Nations in 1923, and 1928, and 1930, at which duplicate samples of some hun-

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dreds of sera were tested by the serologists who had devised diagnostic tests for syphilis, each serologist performing his own test.

Subsequently, the first attempt in the United States at determining the relative values of various techniques was made in 1934-1935, under the joint auspices of the United States Public Health Service and the American Society of Clinical Pathologists. Still later, another evaluation study was carried out in 1937, and others followed in 1938, 1939, and 1940, the scope of participation having been broadened to include not only laboratories of the originators of tests but also most state laboratories and some municipal laboratories as well.

As a result of these comparative studies it has been shown that there are at least five serodiagnostic tests for syphilis which are satisfactory in both sensitivity and specificity as long as the technique of the originator is strictly adhered to. However, the studies also showed that marked variations were obtained by laboratories using supposedly the same procedure when the directions for performance given by the originator of a test were not strictly followed.

As an outgrowth of these evaluation studies, critical reviews of procedures have been stimulated, with the result that substandard or obsolete techniques have been abandoned and emphasis has been placed on the importance of strict adherence to all the minor technical details of a described procedure. The net result has been a marked improvement in the sensitivity and specificity of the serodiagnostic tests for syphilis routinely carried out in the participating laboratories.

THE PROBLEM IN ALABAMA

For many years the Bureau of Laboratories of the Alabama State Board of Health has attempted to attain uniformity of procedure in its central and branch laboratories by various means, including the use of check specimens. Efforts have also been made along this line in cooperation with hospital and private clinical laboratories within the state.

Check specimens for the evaluation of serologic tests for syphilis have been somewhat of a problem, however, since completely authenticated specimens from known syphilitic and nonsyphilitic persons, in numbers necessary for adequate evaluation, such as have been furnished in the interstate evaluations of the United States Public Health Service and the intrastate evaluations conducted in Maryland[1] in 1939-1940, and in Georgia[2] in 1940, have not been available. As a substitute for such specimens, and as a means of making possible continuous weekly checks of serodiagnostic tests for syphilis, the following procedure has, therefore, been used for some time in Alabama.

METHOD

The primary assumption of this method of evaluation is that certain serodiagnostic tests for syphilis have already been demonstrated to be satisfactory by the United States Public Health Service. Consequently, evaluation of the ability of a procedure to detect diminishing amounts of reagin in diluted specimens constitutes an adequate check on the sensitivity of the test in the laboratory being checked. Sufficient negative sera are included over a period of time to evaluate specificity.

Blood specimens received in the central laboratory for serologic test for syphilis are first tested routinely by means of the presumptive Kahn test. Sera that give a negative result by this test, which are to be used in the preparation of check specimens, are set aside and later retested by the presumptive Kahn test, using the serum undiluted and in a 1:5 saline dilution, to assure against technical errors and prozones. Sera reacting negatively to these two tests are pooled for use in preparing check specimens. Diagnostic sera giving any degree of reaction in the presumptive Kahn test are subjected to the standard Kahn test; sera giving a strongly positive reaction with the standard Kahn test are also pooled for use in preparing check specimens. The pooled negative sera and the pooled positive sera are Seitz-filtered separately, to free them from bacteria and undesirable precipitates; such filtration has caused no difference in reactivity so far as parallel specimens have indicated.

Eight dilutions of positive in negative serum—1:2, 1:4, 1:5, 1:8, 1:10, 1:16, 1:20, and 1:30—are prepared in sterile flasks. These dilutions are most easily prepared by making basic dilutions of 1:2 and 1:5, and preparing the higher dilutions from these. These dilutions are transferred to sterile, corked tubes, serially numbered in groups of 10; the numbers on the tubes purposely bear no relationship to the dilution of serum; the ninth and tenth tube in each group are for the inclusion of one tube of undiluted positive serum and one tube of undiluted negative serum. Approximately 200 c.c. of negative serum and 50 c.c. of positive serum have been found ample for preparing the 10 check specimens for 12 to 15 laboratories. Sets of tubes are assembled, and a sheet of instructions as to the inactivation and the method of reporting results is included, and the specimens are mailed to the various laboratories simultaneously. When the results are received, they are tabulated as shown in Table I; errors are checked and a copy of the composite results is sent to each serologist.

INTERPRETATION OF REPORTED RESULTS

From Tables I and II it appears that any laboratory which obtained a 2+ or stronger reaction in its diagnostic test with these particular sera in a 1:5 dilution was running a satisfactorily sensitive test, and for that reason a line is drawn across the tables after the 1:5 dilution. Any laboratory which failed to get a positive reaction in this dilution was considered as conducting an insensitive test. If any error is made in the choice of this critical dilution, it is believed that it is on the side of conservatism, since it is thought that an effort to obtain maximum sensitivity may result in a certain number of false positives, i.e., some loss in specificity.

In Table I it appears that laboratories 1 and 2 underread or overread the sera diluted 1:16 and 1:20, and that laboratories 2 and 6 performed supersensitive presumptive Kahn tests. It also appears that laboratory 7 underread the 1:4 or overread the 1:5 dilutions of its standard Kahn test, whereas laboratory 9 performed an undersensitive presumptive Kahn test.

In Table II, on the particular day these specimens were examined, laboratories 13, 14, and 15 conducted undersensitive complement fixation tests, and laboratory 13 an undersensitive standard Kahn test. In laboratory 12

TABLE I

CHECK SEROLOGIC SPECIMENS

Results Obtained in 9 Laboratories, All Using the Same Techniques and Antigens

Date: July 23, 1940.

LAB. NO.	DILUTION	LAB. 1		LAB. 2		LAB. 3		LAB. 4		LAB. 5		LAB. 6		LAB. 7		LAB. 8		LAB. 9	
		PRS. KAHN	ST. KAHN	PRS. KAHN	ST. KAHN	PRS. KAHN	ST. KAHN	PRS. KAHN	ST. KAHN	PRS. KAHN	ST. KAHN	PRS. KAHN	ST. KAHN	PRS. KAHN	ST. KAHN	PRS. KAHN	ST. KAHN	PRS. KAHN	ST. KAHN
315	Undiluted +	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
317	1:2	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	3
318	1:4	4	4	4	4	4	3	4	3	4	3	4	3	4	3	4	3	3	3
311	1:5	4	3	4	3	4	2	Broken		4	3	4	3	4	4	4	3	3	2
319	1:8	4	1	4	1	3	1	4	1	4	1	4	1	4	2	3	1	2	1
312	1:10	3	±	4	1	3	1	4	1	4	1	4	±	3	1	3	-	2	1
320	1:16	2	-	2	-	2	-	4	-	3	-	3	-	3	-	2	-	1	-
313	1:20	3	-	4	-	2	-	4	-	3	-	3	-	2	-	2	-	-	
314	1:30	1	-	2	-	-		1	-	1	-	3	-	1	-	1	-	-	
316	Undiluted -	-	-	-		-		-		-		-		-		-		-	
Comments:																			
Supersensitive test				X								X							
Undersensitive test																		X	
Underreading		1:16		1:16										1:4					
or																			
Overreading		1:20		1:20										1:5					
Technical error																			

TABLE II

CHECK SEROLOGIC SPECIMENS

Results Obtained on the Same Specimens as in Table I in 6 Laboratories, Using Varying Techniques and Antigens

Date: July 23, 1940.

LAB. NO.	DILUTION	LAB. 10		LAB. 11		LAB. 12				LAB. 13		LAB. 14		LAB. 15
		MICRO EAGLE	ST. KAHN	PRS. KAHN	ST. KAHN	PRS. KAHN	ST. KAHN	MICRO EAGLE	EAGLE COMP. FIXATION	ST. KAHN	WASSER-MANN	ST. KAHN	WASSER-MANN	KOLMER
315	Undiluted +	4	4	4	4	4	4	4	4	4	4	4	4	4
317	1:2	4	4	4	4	4	4	4	4	4	4	3	3	3
318	1:4	3	3	4	4	4	4	4	4	2	2	2	-	-
311	1:5	3	3	4	4	4	4	4	3	1	1	2	1	-
319	1:8	2	1	4	3	3	3	3	1	-	-	-	-	-
312	1:10	1	1	4	3	4	3	3	1	-	-	-	-	-
320	1:16	1	-	4	2	2	1	2	±	-	-	-	-	QNS
313	1:20	-	-	4	1	2	3	2	±	-	-	-	-	-
314	1:30	-	-	1	-	-	-	-	-	-	-	-	-	-
316	Undiluted	±	-	-	-	-	-	-	-	-	-	-	-	QNS
Comments: Supersensitive test														
Undersensitive test										X	X		X	X
Underreading or Overreading						1:8 1:10	1:16 1:20						1:4 1:5	
Technical error		X												

underreading in the lower dilution or overreading in the higher dilution seems to have occurred in the 1:8 or the 1:10 dilution in the presumptive Kahn test, and in the 1:16 or the 1:20 dilution in the standard Kahn test. The same error seems to have been made in laboratory 14 in the 1:4 and 1:5 dilutions in the Wassermann test. In laboratory 10 a certain degree of false positive reaction was obtained in the negative control serum with an Eagle flocculation test.

SUMMARY

A plan is presented for the conduct of serologic check testing which is being used with success by the Bureau of Laboratories of the Alabama State Department of Public Health. The plan has several important advantages. It is inexpensive. It utilizes materials available in the laboratory every day. It enables checking of performance at weekly intervals throughout the year. By including enough frankly negative sera with those that contain varying amounts of reagin, it checks on both specificity and sensitivity. It readily indicates errors in technique, and it encourages revisions in procedure.

CONCLUSIONS

1. Intrastate evaluations of the performance of serologic tests for syphilis are of great value, not only to the participating laboratories but also to the physicians and to the public.

2. To be of greatest usefulness the evaluation of performance of serodiagnostic tests for syphilis must be made at frequent intervals.

3. A practical method for conducting intrastate evaluations of serologic tests for syphilis, based on the "horizontal method of analysis," has been described.

4. The method described is economical and checks both specificity and sensitivity.

5. On the basis of check testing by the method described, provision is made to assist any laboratory in improving its performance of serodiagnostic tests for syphilis through inspection, and the instruction of serologists in the Bureau of Laboratories of the State Department of Health.

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A SIMPLE MODIFICATION OF WELTMANN'S COAGULATION REACTION*

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NUMEROUS investigators abroad, and Kraemer,[1] Levinson, Klein, and Rosenblum[2, 3] in this country, have established the value of Weltmann's serum coagulation reaction for diagnosis and prognosis. However, the original technique is too cumbersome to be used as routine procedure. It involves the frequent preparation of ten solutions of calcium chloride, a set-up of at least ten test tubes, and a water bath. The modification outlined here requires only one test tube, one standard solution of calcium chloride, and 0.1 ml. of serum.

TABLE I

TUBE	I	II	III	IV	V	VI	VII	VIII	IX	X
Weltmann's method										
$CaCl_2$/1,000	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
mg. $CaCl_2$/5 ml.	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5
Modification										
ml. of 1% $CaCl_2$	0.5	0.45	0.4	0.35	0.3	0.25	0.2	0.15	0.1	0.05
		Exudative zone Shift to left Short band				Normal zone		Fibrotic zone Shift to right Long band		

Principle.—A 1:50 dilution of normal human serum with distilled water does not coagulate on heating unless a bivalent electrolyte, such as calcium chloride, is added to make a concentration of 0.4 to 0.5 per thousand. Sera from conditions where exudation prevails, such as pneumonia, acute rheumatic fever, and exudative tuberculosis, require an increased concentration of electrolyte to one per thousand and more. On the other hand, sera from cases where fibrosis and liver damage are prominent, such as fibrotic tuberculosis, subacute atrophy of the liver, cirrhosis of the liver, and septic conditions, coagulate with very low concentrations (0.1 to 0.3 per thousand).

Weltmann placed into ten test tubes 4.9 ml. of solutions, ranging from one per thousand to 0.1 per thousand of crystalline calcium chloride. The integral amounts to 0.1 per thousand or 0.5 mg. calcium chloride per 5 ml. This amount is contained in 0.05 ml. of a 1 per cent solution of calcium chloride, which is the standard solution used in the modification.

Technique.—Into a medium-sized test tube (about 6 by 0.5 inches) place 4.9 ml. of distilled water and 0.1 ml. of clear nonhemolytic serum. Add from a 0.1 ml. pipette 0.05 ml. of 1 per cent calcium chloride (equal to 0.5 mg.). Shake and bring to a boil over a small flame. Cool and repeat the procedure until flocculation has taken place. Note the milligrams necessary to produce floccula-

*From the Arthritis Clinic and Department of Laboratories, White Memorial Hospital, College of Medical Evangelists, Los Angeles.

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tion. Table I gives the conversion into the corresponding calcium chloride concentration and also for comparative purposes the number of the tube of Weltmann's original reaction. Violent boiling should be avoided because it may lead to foaming and loss of fluid. The observation for flocculation should be made after cooling. In practice it is not necessary to proceed by single units of 0.05 ml.

The first boiling can be done after the addition of 0.15 ml. of calcium chloride. This is still lower than the concentration necessary for the flocculation of normal serum and is characteristic of fibrotic processes. If the serum is from known exudative cases, such as pneumonia or active tuberculosis, the test can be started by the addition of 0.25 ml., the upper normal range. After one has acquired enough experience, he can judge by the amount of turbidity after the first boiling approximately how many more units of calcium chloride will still be required, thus shortening the test.

A slight increase in dilution of serum occurs by the addition of calcium chloride to the 1:50 dilution. For the whole range of the Weltmann test the error is not greater than 1 to 10 per cent, which is compensated by the loss of fluid through boiling. A comparison between the original Weltmann test and the modification in 61 cases showed an average difference of less than one tube. In severe exudative cases, such as pneumonia, where flocculation may occur far beyond the range of 1:1,000 (Weltmann's tube I) 15 to 20 tubes would have to be set up. By the modification the severest stages are conveniently and quantitatively determined by the simple addition of the number of units of the calcium chloride necessary for complete flocculation. The results of the test in 307 cases are summarized in Table II.

TABLE II

CONDITION	NUMBER OF CASES	MAXIMUM (ML. OF 1% $CaCl_2$)	MINIMUM (ML. OF 1% $CaCl_2$)	AVERAGE (ML. OF 1% $CaCl_2$)
Pneumonia	31	1.0	0.3	0.59
Bronchitis, influenza	6	0.45	0.2	0.35
Pulmonary tuberculosis	29	0.65	0.15	0.36
Tonsillitis	14	0.45	0.2	0.26
Inflammation of gall bladder, urinary bladder, sinuses, prostate	34	0.55	0.15	0.3
Fibroid tumors	5	0.5	0.2	0.35
Carcinoma	7	0.5	0.15	0.26
Pregnancy, post partum	32	0.45	0.15	0.25
Arteriosclerosis, hypertension, chronic myocarditis and circulatory disorders	23	0.7	0.2	0.25
Appendicitis	16	0.65	0.15	0.28
Chronic gonorrhea	5	0.55	0.2	0.32
Syphilis	9	0.4	0.2	0.28
Goiter	7	0.3	0.15	0.27
Gout	6	0.35	0.3	0.33
Infectious, rheumatoid arthritis	48	0.55	0.15	0.36
Osteo-arthritis	35	0.45	0.15	0.27

The cases were not selected as to stage or complications. The maximum values indicate the degree of exudation which is brought about primarily either by the disease, such as pneumonia or active tuberculosis, or by the secondary tissue destruction and inflammation, such as occur in carcinoma. The minimum

values are characteristic for subsidence of exudation, development of fibrosis, or a combination of both processes in the chronic stages.

Of the modifications which have been published abroad, Havas' micromethod retains the cumbersome original technique. The only advantage is reduction of the necessary reagents and serum to one-tenth of that required by the Weltmann method. Teufel's modification uses the same principle as described in this paper, but employs only 0.5 per cent calcium chloride. The error of dilution is, therefore, doubled. To deliver the calcium chloride he uses special droppers which are expensive, not easily available, and introduce another error because they do not deliver the exact unit of calcium chloride that is required.

I wish to express my appreciation to Dr. Orlyn B. Pratt, director of the laboratories, Department of Pathology, White Memorial Hospital, College of Medical Evangelists, for providing the facilities of the laboratory for this work.

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NITRIC AND SULFURIC ACIDS, A COLORLESS CLEANING MIXTURE FOR GLASSWARE

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MANY scientific workers are apparently unaware of the useful properties of a mixture of nitric and sulfuric acids for cleaning glassware.

The most commonly used cleaning mixture consists of concentrated sulfuric acid containing dissolved sodium or potassium dichromate (or better, chromium trioxide). When freshly prepared, this makes an excellent cleaning solution, but it rapidly becomes spent with use, turning green owing to the reduction of the chromium trioxide by organic materials on the glassware. More of the chromium compound may then be added, but the mixture soon becomes so thick and pasty that it must be discarded. This mixture has the further disadvantage of having a very dark color, so that it is extremely difficult to find small objects and to remove them from the bath. Furthermore, it is claimed[1] that traces of chromium which adhere to the glass can only be removed by very extensive washing, and, therefore, may contaminate biological materials which subsequently come in contact with the glass. This is especially true in the case of fritted glass filters. Traces of chromium have a strongly toxic action on many microorganisms.[2]

A mixture of concentrated sulfuric and nitric acids has none of these disadvantages. A cold mixture containing about 10 per cent of nitric acid by vol-

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ume is very effective in removing traces of grease from glassware (such as pipettes or microscope slides) after a few hours' immersion. When hot, the mixture is extremely effective in oxidizing large amounts of organic material (such as resins, oils, or dried biological fluids) adhering to the glass. In so doing, it often takes on a brown color due to partly oxidized organic matter and liberated nitric oxide, but upon continued heating to complete oxidation, the color of the bath will return to light yellow or light yellow green. Too much organic matter will consume the nitric acid, in which case more can be added as needed. It should be stirred in well, preferably after the mixture has cooled.

If much cleaning is done a convenient method of use is to maintain a bath of sulfuric acid in a large evaporating dish or other acid-resisting container on a gas range or electric hot plate under a hood, and to add a small amount of nitric acid with thorough stirring at the beginning of each day's work, before turning on the heat. A duriron dish is extremely desirable, and avoids the danger of breakage which is always present with porcelain or glass containers. The nitric acid should be well stirred into the cold sulfuric acid, otherwise it will form a layer on the surface and will evaporate rapidly as the bath becomes hot. Such a cleaning bath may be maintained for several months, and need only be discarded when the sulfuric acid has become viscid by the slow accumulation of inorganic salts from the oxidized materials.

The mixture has the disadvantage of fuming somewhat when exposed to the air, especially when heated; hence to avoid breathing the irritating fumes it should be used in a hood with a good current of air. As with the dichromate-sulfuric acid mixture, great care should be taken to avoid splashing the mixture on the hands or clothing, since severe burns may result. Steel crucible tongs are useful in removing glassware from the bath, and such glassware should be well cooled under the hood before rinsing with water. If exposed to the air for several days without heating, the mixture will absorb atmospheric moisture and increase in volume, but the absorbed water rapidly evaporates when the bath is heated again.

The use of chlorates or perchlorates in sulfuric acid cleaning baths has sometimes been proposed, but such mixtures are *extremely dangerous* on account of the explosion hazard and should always be avoided. Sodium or potassium nitrates have also been used, but they have no advantages over nitric acid. The addition of any solid salt will make the bath viscid and shorten its useful life.

Care should, of course, be taken not to heat the mixture so hot that white fumes of sulfuric acid arise, since this will cause a large loss of the acid. If enough nitric acid is present, very gentle heating is usually sufficient to oxidize most organic materials on the glass.

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FREEZING OF TISSUES WITH "DRY ICE" FOR MICROTOME SECTIONING OF THE ENTIRE BRAIN*

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IN RECENT YEARS, solid carbon dioxide ("dry ice") has been used in freezing tissues preparatory to sectioning and has been found to have many advantages over the gas. Lindsay and co-workers,[1] and Dunn[2] described "dry ice" attachments for freezing small blocks of tissue, which can be used with the standard freezing microtome. Mason[3] devised a method of using the rocking microtome for sections frozen with carbon dioxide gas.

We have constructed a "dry ice" attachment by which coronal sections of the entire cerebrum may be easily frozen and cut on the larger sledge type celloidin microtome (Minot-MacCallum). For those laboratories which possess a microtome that can accommodate sections 4 by 4 inches (Minot), a smaller attachment was constructed. With this attachment, a coronal section of one cerebral hemisphere, the basal ganglia, whole animal brains, infant brains, kidneys, spleens, etc., may be sectioned.

FREEZING APPARATUS

The freezing attachment consists essentially of (1) a metal plate upon which the slab of tissue is frozen, and (2) a means of keeping the "ice" which in turn chills the tissue, at the same time anchoring the tissue to the plate. Freezing will not occur unless the "ice" is in contact with the plate. After the "ice" is removed, the hardening process continues from the cold plate. With a thin plate, the freezing process is rapid; a thicker plate slows the freezing process but retains the cold longer.

The freezing plate of the larger apparatus consists of sheet aluminum, 1/8 inch thick and measuring 6½ by 5 inches. This is screwed to a bakelite box,† measuring $6\frac{13}{16}$ by $5\frac{5}{16}$ by $2\frac{5}{16}$ inches, of which it becomes the roof. The end of the box is cut away, and a wooden drawer is built to fit snugly into the box through this end. The drawer (Fig. 1, *D*) contains a slab of "dry ice" (*I*) which is forced against the aluminum plate (*P*) by a false bottom (*I'*) mounted on spiral springs (*S*). The springs slide on thin metal tubes (*T*), each of which surrounds a bolt (*B*) which is fastened to the floor of the drawer by a nut (*N*). At the free end of the bolt, a washer (*K*) and nut (*M*) limit the upper excursion of the false bottom. The washer (*W*) helps keep the spring in place. The bakelite box (*X*) is screwed to the object clamp supporting base of the microtome.

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†Molded Bakelite Meter Box, No. K10531, sold by the Lafayette Radio Company, 100 6th Avenue, New York City.

To anchor the tissue more firmly to the freezing plate, moistened filter paper may be interposed, or deep grooves may be cut into the plate.

The smaller apparatus consists simply of an electrical conduit box, 4 by 4 by 1⅝ inches, and a false bottom of hard rubber mounted on springs, as in the

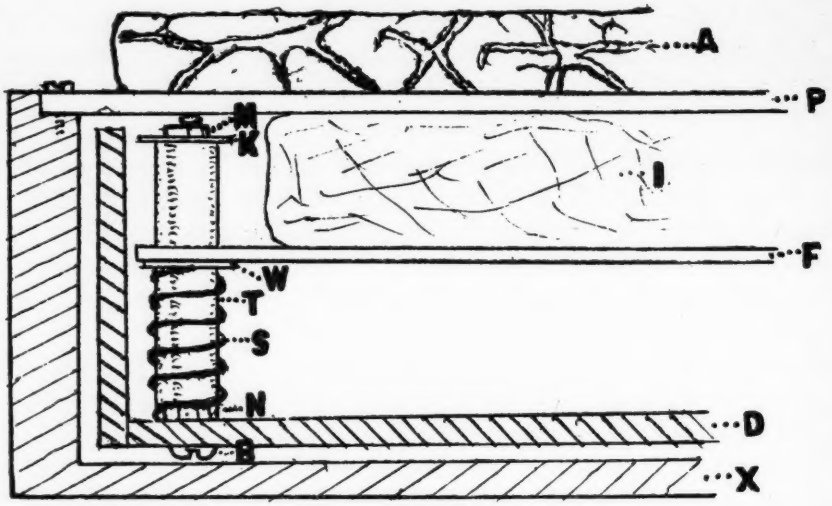

Fig. 1.—Diagram of freezing attachment. See text for explanation of letters.

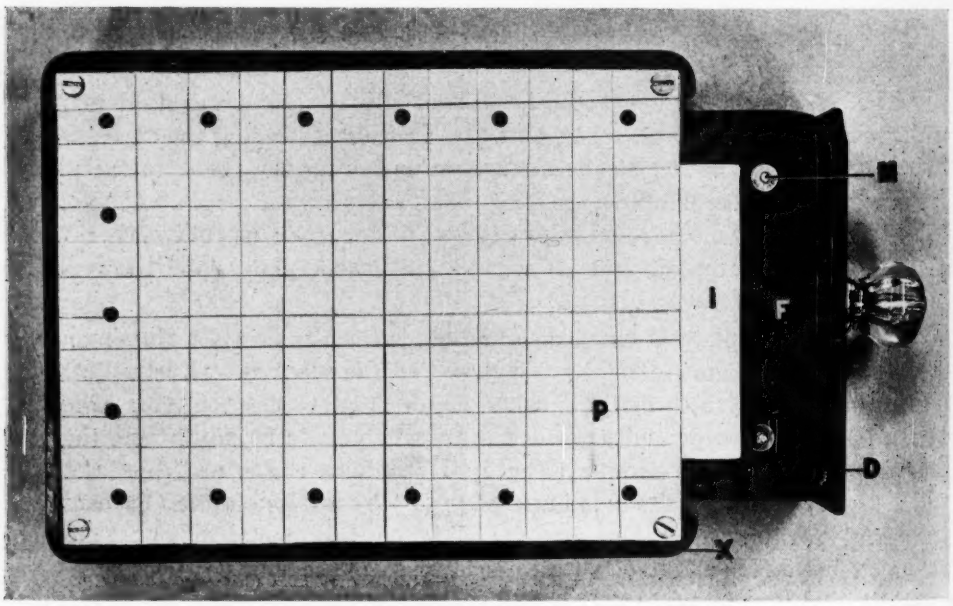

Fig. 2.—Top view of larger freezing attachment, showing aluminum freezing plate and partly open drawer containing a slab of "dry ice."

larger apparatus. The cover of the box is used as the freezing plate. No drawer is used in this case. When the tissue is frozen, the freezing plate is slipped off and the "ice" is removed. The plate is then screwed in place, the box is placed in the object clamp of the microtome, and cutting can begin.

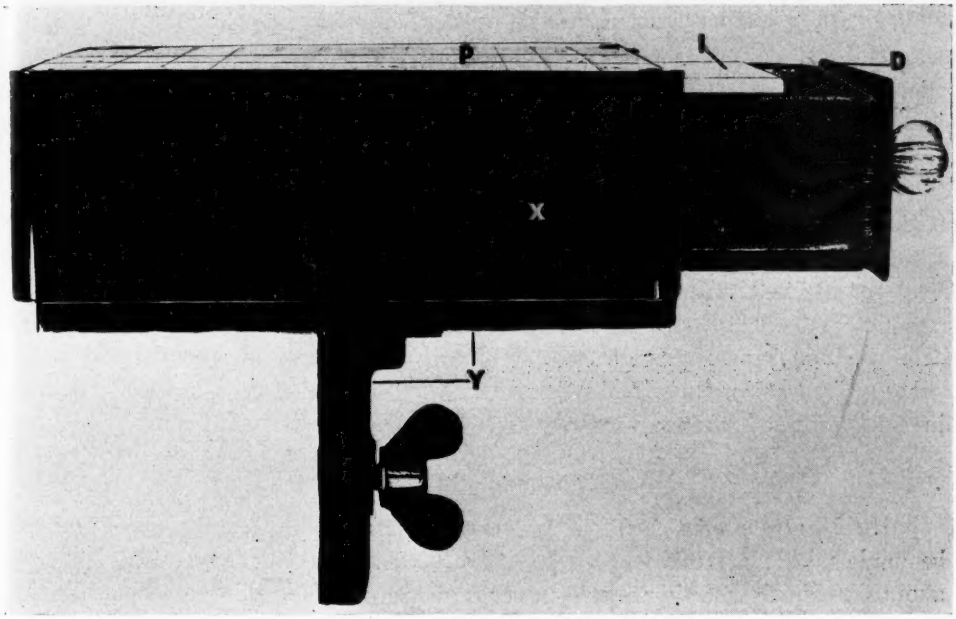

Fig. 3.—Side view of larger freezing attachment, showing bakelite box attached to object clamp supporting base of microtome.

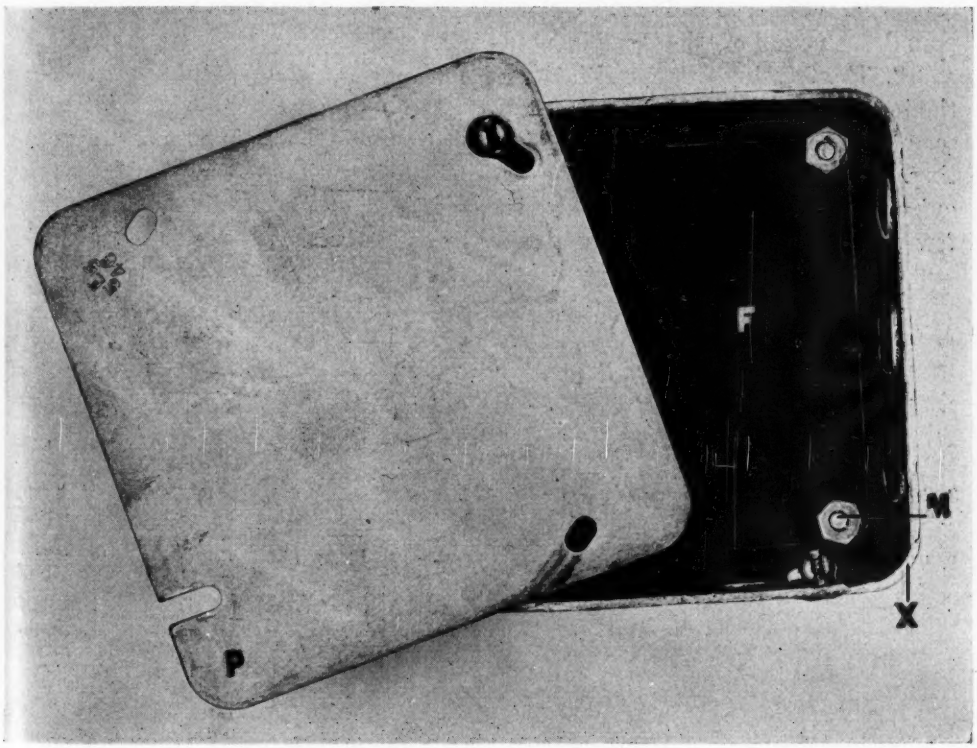

Fig. 4.—Top view of smaller freezing attachment, showing partly removed freezing plate and false bottom within conduit box.

TECHNIQUE

The brain is fixed for ten days in 10 per cent formalin and then cut into slices 1 cm. thick. A slab of brain is placed on the freezing plate and "dry ice" is placed in the freezing chamber. A glass dish is inverted over the tissue. The freezing process varies according to room temperature. Ordinarily the "ice" is removed in about five minutes. The hardening process continues for a few minutes after the "ice" is removed. The surface of the section should be blotted free of water to prevent the formation of ice. Sections 20 to 30 microns thick may be obtained. The sections are brushed into a pan of water where they are straightened out without difficulty. They are easily transferred from dish to dish during the staining process by means of a large perforated flat spoon previously described by Lubinsky.[4] Further technical details are well outlined in Christeller's *Atlas*[5] and may be found in Abbott's translation.[6]

ADVANTAGES OF "DRY ICE" METHOD

1. The attachment for freezing coronal sections of the entire cerebrum can be made with materials costing less than $2.00. The smaller attachment costs about 30 cents. This is a small fraction of the price for a large carbon dioxide gas attachment. No special microtome is required. Using the principles described, one may devise an attachment to suit any microtome.

2. When carbon dioxide gas is used for freezing coronal sections of the cerebrum, a large tank is soon exhausted. "Dry ice" costs only a few cents a pound. A pound of "ice" can be used for freezing two or more coronal sections.

3. The freezing process with "dry ice" requires about five minutes and sectioning may proceed for ten to fifteen minutes. Because of this long interval, some serial sections may be obtained and little tissue is lost in preparatory cutting. According to Christeller, the freezing process with carbon dioxide gas requires twenty to thirty minutes for large sections, and with this method only a few minutes are available for sectioning before refreezing is necessary.

4. The attachment requires little space and is easily transported.

We wish to acknowledge our indebtedness to Dr. Tracy Putnam at whose suggestion this work was undertaken.

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AN EFFICIENT OXYGENATOR FOR BLOOD*

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IN AN EARLIER publication[1] a method of introducing oxygen into blood was described. It consisted of producing a thin film of blood on the inner surface of a vertical revolving cylinder. The apparatus was not capable of introducing oxygen rapidly enough for the experimental purposes for which it was designed. In the present report a larger cylinder of improved design, capable of introducing 60 per cent more oxygen into blood, is described.

The principal feature of the apparatus is a vertical steel cylinder, 24 inches in length and 8½ inches in inside diameter (Fig. 1). The cylinder is supported by two sets of roller bearings with circular racers. These allow the cylinder to revolve around a vertical axis. The racers are in turn supported by a vertical steel pipe 4 inches in diameter, with a wall ⅝ inch thick. The pipe is 40 inches high, mounted on a steel base, 12 by 20 inches in area. Revolution of the cylinder is accomplished by a one-quarter horse power A.C. motor, attached by a pulley and V-belt to a vertical segment of a steel sphere covered with hard rubber (Fig. 1, side view). The convex surface of the segment of a sphere is pressed by a strong steel spring against the beveled edge of a steel collar that encircles the revolving cylinder. Revolution of the spherical segment produces revolution of the cylinder by this sliding friction drive that has a contact of about one-half inch. The spherical steel segment can be moved about a horizontal axis in such a way as to shift the frictional driving surface from the center to the periphery of the spherical segment. This shift is capable of varying the speed from 60 to 360 r.p.m. This is an improvement upon the previous method[1] of changing the rate of revolution of the cylinder by varying the armature resistance of a D.C. motor.

In the old apparatus great difficulty was experienced in producing a rust-proof inner surface for the cylinder. In the present apparatus a "Micarta"† cylinder ⅛ inch thick fits snugly within the steel cylinder and provides a smooth, rust-proof surface for the film of blood. The lower end of the cylinder is tapered from within outward to a knife edge. A chrome-plated copper cup slides up over the lower end of the cylinder, overlapping the cylinder by 1⅛ inches. The space between the lower, sharp end of the cylinder and the inner wall of the stationary cup is less than 1 mm. The lower part of the cup is funnel-shaped and terminates in a central aperture ¾ inch in diameter, from which a tapered

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†A plastic made by Westinghouse Electric Company.

collar extends downward. Over this tapered collar is slipped the neck of a glass cup with a similar taper. The junction between the ground-glass neck and the metal collar is sealed with stopcock grease, and the glass cup is held in position by two small springs extending from the glass cup to the bottom of the metal cup. Both the metal and the glass cups are jacketed separately to permit the circulation of water between the walls in order to maintain the blood at the desired temperature.

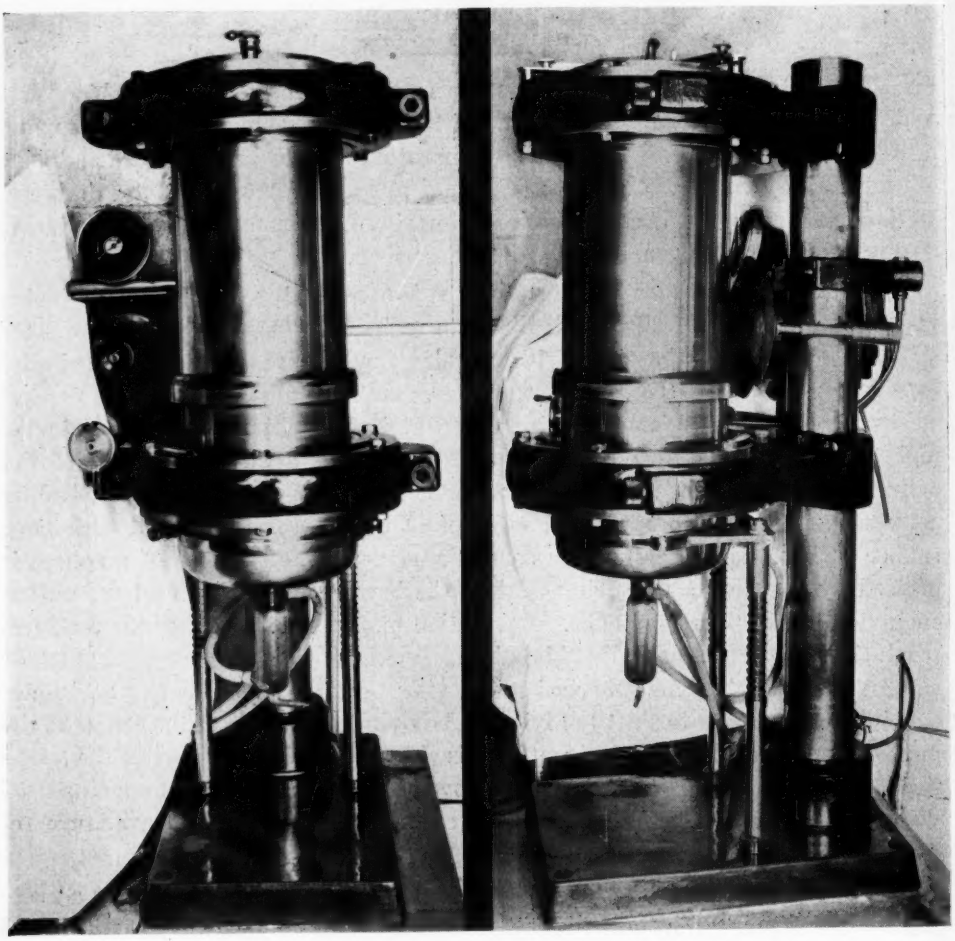

Fig. 1.—Photographs of oxygenator. Front and side views.

The greater portion of the space within the revolving cylinder is occupied by a closed, stationary cylinder, as in the previous model. Through the center of this closed cylinder passes a tube which is used to convey a mixture of oxygen and carbon dioxide at a rate of 5 liters per minute to the lower portion of the inside of the apparatus. The oxygen mixture then diffuses upward in close contact with the inner wall of the revolving cylinder. When using the apparatus for perfusion, a mixture of 95 per cent oxygen and 5 per cent carbon dioxide is used so as to maintain an approximately normal carbon dioxide content in the blood.

Blood is introduced at the top of the cylinder through a stainless steel tube having a 2.5 mm. bore. The tube is curved and its end is flattened so that it delivers a fanlike stream of blood against the inner wall of the revolving cylinder in the direction of revolution and tangential to the curved surface of the cylinder. The tube is locked in position on the inner stationary cylinder, but is detachable for cleaning. When blood is pumped through this inlet tube onto the upper part of the inner surface of the cylinder while it is revolving, a film of blood is established in a horizontal band about 4 inches high around the upper part of the cylinder. At the lower margin of this band the film of blood breaks up into vertical rivulets of blood approximately 0.5 cm. in width, spaced at intervals of 2 cm. Under these circumstances relatively small amounts of oxygen are introduced into the blood. Consequently, it was found necessary to prime the cylinder with blood in order to establish a film. This priming can probably be accomplished in a variety of ways. It was found simplest to wipe the vertical length of the cylinder with a gauze sponge while the cylinder is revolving and the blood is flowing in at the top. Once the film is established, it will remain as long as the cylinder is revolving and blood is flowing in at the top; but both the revolution of the cylinder and the flow of blood may be stopped simultaneously for brief intervals without the loss of the film.

The optimum rate of revolution of the cylinder is in the neighborhood of 250 r.p.m. Faster rates have not facilitated the introduction of oxygen into blood. With slower rates the film of blood on the inner surface tends to split up into rivulets when the flow of blood through the oxygenator is small. Foaming occurs only if air enters the tubing through which blood is being pumped into the oxygenator.

INTRODUCTION OF OXYGEN

The amount of oxygen that can be added to blood by this apparatus was determined in the following manner: Venous blood was continuously withdrawn from the superior vena cava of an animal and pumped into the top of the oxygenator. The oxygenated blood was pumped back from the cup at the bottom of the cylinder into the femoral artery in a central direction. The pumps used were of the constant injection roller type as modified by DeBakey[2] and driven by an electric motor.[3] The rate of the pumps was varied by an armature resistance, and the speed of the motor was read directly on a speedometer. The output of the pumps was calibrated by measuring the delivery of blood at different rates of revolution after the experiment. Cats were used in five instances, Nos. 1 to 5, inclusive, in Table I; and dogs in six instances, Nos. 6 to 11. Several weeks prior to the experiments on the cats, the ribs overlying the pericardium were excised and the pericardium was sutured to the chest wall just beneath the skin. The pulmonary artery could thus be exposed and clamped without producing an open pneumothorax. In all the cats the pulmonary artery was completely occluded. During the period of occlusion the circulation of blood through the animal was maintained by the mechanical pumps, and the respiratory function of the lungs was performed by the oxygenator.[3] To obtain a larger flow of blood dogs were used in the last six observations in Table I. In these animals

the pulmonary artery was not occluded. In observations Nos. 8, 9, 10, and 11, the dogs were allowed to inhale from a spirometer filled with 85 per cent nitrogen and 15 per cent oxygen in order to decrease the saturation of the venous blood with oxygen. In all the experiments sufficient heparin was given intravenously to prevent coagulation of the blood. Simultaneous samples of blood were withdrawn under oil from the tubes leading to and from the oxygenator. The samples were immediately analyzed for oxygen and carbon dioxide content and oxygen capacity. The determinations were made in duplicate. The greatest difference between duplicate samples in oxygen content was 0.15 volumes per cent, and in oxygen capacity it was 0.20 volumes per cent.

TABLE I

OBSERVATION NO.	OXYGEN CAPACITY (VOL. %)	SATURATION		OXYGEN ADDED TO BLOOD (C.C./100 C.C.)	BLOOD FLOW (C.C./MIN.)	OXYGEN ADDED TO BLOOD (C.C./MIN.)	CARBON DIOXIDE IN OXYGEN (%)
		% BEFORE	% AFTER				
1	16.75	57	99	6.88	130	8.94	5
2	17.01	36	99	10.57	240	25.37	0
3	14.51	58	93	5.19	255	13.23	6
4	14.76	72	95	3.52	270	9.50	5
5	14.23	41	99	8.35	285	23.80	5
6	17.32	72	96	4.11	370	15.21	5
7	21.90	73	92	4.24	375	15.90	5
8	21.19	48	82	7.39	380	28.08	5
9	22.03	40	72	7.04	395	27.81	5
10	17.13	32	64	5.41	420	22.72	7
11	21.87	64	95	6.71	455	30.53	5

The results are arranged in Table I in order of increasing blood flow. It can be seen that up to rates of 375 c.c. per minute the blood leaving the oxygenator is adequately saturated with oxygen at varying degrees of venous saturation. In three instances (Nos. 8, 9, 10) the saturation of the blood leaving the oxygenator was below 92 per cent. In one of these, No. 10, the blood was only 64 per cent saturated, but there was marked venous unsaturation; a gas mixture of 93 per cent oxygen and 7 per cent carbon dioxide was passed through the oxygenator instead of the usual mixture of 95 per cent oxygen and 5 per cent carbon dioxide. In this experiment the carbon dioxide content of the blood leaving the oxygenator was 51.23 volumes per cent, whereas in the other ten instances the carbon dioxide content varied between 42.83 volumes per cent and 30.59 volumes per cent. In Nos. 8 and 9 there was also a marked unsaturation of venous blood, and the flow was relatively large.

HEMOLYSIS

To maintain normal function when perfusing an isolated kidney it is probably important to avoid hemolysis in the blood used for perfusion.[4] Similarly, in the perfusion of the entire body of an animal it must be equally important to avoid hemolysis. To determine the degree of hemolysis produced by the oxygenator, blood was allowed to flow into the apparatus by gravity from a 250 c.c. burette at a rate between 250 and 350 c.c. per minute (the range of blood flow

used to perfuse cats of average size). Blood was drained from the glass cup at the bottom of the oxygenator into a flask which was intermittently emptied into the burette which supplied blood to the oxygenator. The total volume of blood used in these observations was approximately 600 c.c. The temperature of the blood was maintained between 37.5° and 38.5° C. by the circulation of warm water through the double walls of the stationary metal and glass cups.

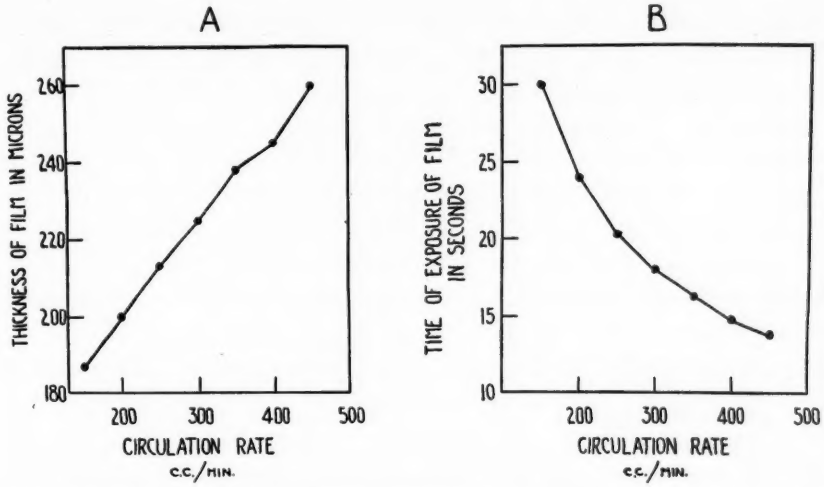

Fig. 2.—*A*, Relation of flow of blood through oxygenator to thickness of blood film. *B*, Relation of blood flow to time of exposure of film.

The blood used was obtained from a dog the day before the observations were made, and was kept in an icebox overnight. Coagulation was prevented by the addition of heparin. At the start of the observations, a sample of blood was placed in a Wintrobe hematocrit tube.[5] The blood was then passed continuously through the oxygenator with the cylinder revolving at 250 r.p.m. Samples of blood were removed and placed in hematocrit tubes every fifteen minutes, until the blood had circulated through the oxygenator for an hour and a quarter. Cells were separated from the plasma in the hematocrit tubes by centrifuging. The colors of the samples of plasma were then compared with the colors of a set of standard tubes of similar diameter containing known dilutions of completely hemolyzed red blood cells. It was found that up to thirty or forty-five minutes the plasma remained practically clear (i.e., faint straw color). From then on there was a steadily increasing rate of hemolysis. At the end of an hour and fifteen minutes of passage through the oxygenator, the plasma had a pronounced reddish tinge.

THE BLOOD FILM

The area available for the filming of blood on the inner surface of the vertical revolving cylinder is 4,000 sq. cm. This represents a 58 per cent increase in area over the oxygenator previously described.[1] The glass cup at the bottom of the oxygenator holds 12 c.c. of blood at an appropriate operating level. The volume of blood on the surface of the revolving cylinder was determined at different rates of blood flow as follows: A known quantity of blood

was placed in a graduated glass cylinder. The blood was then pumped from this cylinder into the copper cup at the bottom of the oxygenator from which it drained down into the glass cup. From the glass cup it was pumped back into the graduated glass cylinder, maintaining at all times a constant level of blood in the glass cup. The level of the blood in the graduated glass cylinder was then read at different rates of blood flow from 150 to 450 c.c. per minute. The volume of blood contained in the circuit at different rates of blood flow was thus determined. The revolving cylinder was then added to the circuit, and the determinations were repeated. In this manner the volume of blood on the sides of the revolving cylinder was determined at varying rates of blood flow. From these figures the thickness of the film and the time of exposure of the blood to oxygen were calculated. For example, 85 c.c. of blood are distributed over the surface of the oxygenator when the cylinder is revolving at 250 r.p.m. and blood is flowing through the apparatus at the rate of 250 c.c. per minute. Under these conditions the film of blood on the inner surface of the revolving cylinder is 213 microns thick, and the average time of exposure of the blood film to oxygen is 20.4 seconds.

The graphs of Fig. 2 summarize the observations made. Each point on the graphs represents the mean of six determinations. It was found that with rates of flow from 150 to 450 c.c. per minute the depth of the blood film was directly proportional to rate of blood flow (Fig. 2*A*). The time the film of blood was exposed to oxygen on the inner surface of the revolving cylinder decreased, but at a progressively diminishing rate, as the flow of blood through the apparatus increased from 150 to 450 c.c. per minute (Fig. 2*B*). Both the greater depth of the film and the decreased time of exposure will tend to interfere with the absorption of oxygen by the blood. Therefore, at any given unsaturation of the blood entering the oxygenator, it is to be expected that, as the rate of blood flow increases, a point will be reached at which the blood leaving the oxygenator will no longer be adequately saturated with oxygen. That such a point has not been reached with degrees of venous oxygen unsaturation within the normal range at rest and a blood flow as great as 450 c.c. per minute is indicated by observation No. 11, Table I.

SUMMARY

An oxygenator capable of introducing as much as 30 c.c. of oxygen per minute into blood has been described. The apparatus is designed to accommodate rates of blood flow from 100 to 500 c.c. per minute. Up to 300 c.c. per minute, the blood leaving the oxygenator will be adequately saturated with oxygen even with marked degrees of venous unsaturation; and at rates of flow up to 450 c.c. per minute if the venous unsaturation is within the normal range. The apparatus is simple in construction and easy to operate. Foaming of the blood does not occur, and hemolysis is slight in periods up to forty-five minutes of operation.

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A DEVICE FOR FIXING THE BRAIN AND SPINAL CORD IN ONE PIECE*

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AFTER removing the brain and spinal cord as a single specimen, it is often found difficult to restore them in their proper relationships. In an ordinary brain jar, or even in a larger receptacle, the spinal cord frequently becomes twisted and its anatomic features are distorted. The following simple apparatus was devised to obviate this difficulty:

A glass tank (the type used for household aquariums are easily obtained and relatively inexpensive), 25 by 10.5 by 10 inches in size, is used as a receptacle for primary fixation. A glass cover is fitted over the top of the tank. A metal framework, consisting of two separate parts, as pictured in Fig. 1, is placed within this container which has been partially filled with 10 per cent formalin. The purpose of the framework is to provide a suitable crossbar at either end of the tank from which a brain may be suspended and hang freely, and two properly placed grooves along which the respective spinal cords may be extended in proper alignment with the given brain.

The framework is constructed from two pieces of galvanized wire, 1/8 inch in diameter and 130 and 95 inches in length, respectively. These are bent in a continuous pattern, as seen in Fig. 2, with the aid of vise, pliers, and hammer. Each division of the framework is 7 inches high and 9 inches wide, and provides a series of crossbars resting on uprights, approximately 3½ inches apart. Each crossbar except the first has two grooves an inch wide and an inch deep which lie approximately 1¼ inches apart. These are the grooves in which the spinal cords are supported. The two divisions of the framework are so devised as to form one continuous pattern when placed end to end in the tank. In this fashion, the complete framework has one ungrooved crossbar available at each end of the tank, from which a brain may be suspended and hang freely by means of a loop of thread or metal hook. The respective cord of each of the brains may then be placed in the two grooves of the framework and thereby kept in good alignment with the brain.

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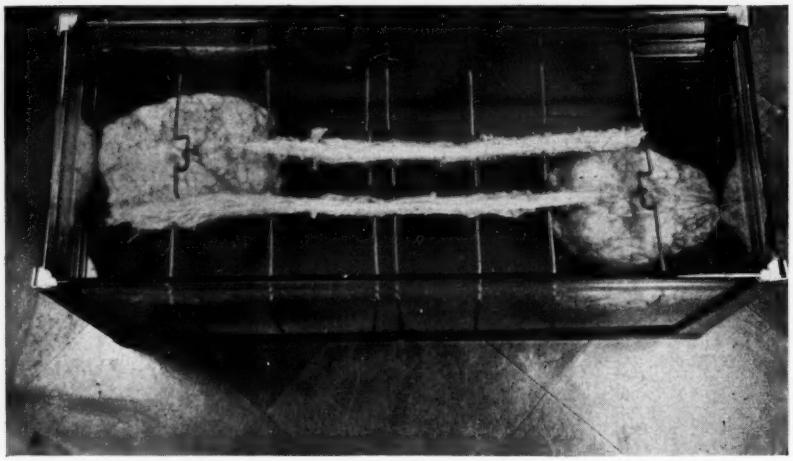

Fig. 1.—Glass household aquarium with brains and their attached spinal cords supported in grooves of wire framework.

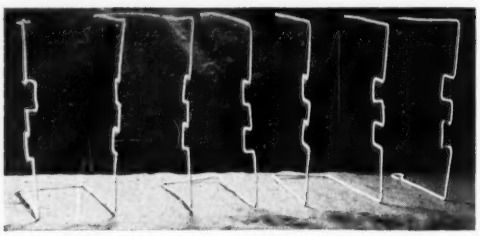

Fig. 2.—Framework consisting of two lengths of wire bent into continuous pattern.

The brain is shifted on the crossbar, so that the spinal cord lying in the groove forms a continuous column with the brain stem. Having the framework in two portions permits the removal of one of the two brains without disturbing the other, or the addition of a fresh specimen to the tank when one is already in place.

A RAPID METHOD FOR STAINING BLOOD SMEARS IN DETERMINING OPSONOCYTOPHAGIC INDICES*

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BECAUSE of the unsatisfactory results obtained from staining blood smears for opsonocytophagic indices with Hasting's stain as recommended by Huddleson and co-workers,[5] many new methods have recently been reported (Cape and Jaffe[2]; Flinn[4]; Bruner and Edwards[1]; Calder[3]). Satisfactory staining for this work, in addition to being simple, should permit accurate enumeration of the organisms ingested by phagocytes. Polymorphonuclear neutrophiles must be readily recognized, and phagocytized bacteria must not be mistaken for cytoplasmic granules. Some of the newer methods (Flinn[4]), while satisfactory, require stains or reagents which are not usually available to most routine laboratories.

The method reported here has been used in this laboratory with considerable success. Citrated blood is mixed with a saline suspension of Brucella cells in the usual manner and incubated at 37° C. for thirty minutes. Blood smears are made and dried quickly to prevent shrinkage of white blood cells. These are then put through the flame of a Bunsen burner four times and allowed to cool. The fixing of smears by flaming is very important and may require practice before the right amount of flaming is obtained. If not flamed sufficiently, smears will tend to wash off the slide, whereas overflaming will result in smears too deeply stained.

The fixed smears are covered with 10 per cent Ziehl-Neelsen carbolfuchsin. This stain is prepared by diluting $\frac{1}{10}$ the Ziehl-Neelsen carbolfuchsin used for tubercle bacillus staining with distilled water and filtering. At the end of two minutes the slides are washed gently with tap water to prevent the dye from precipitating on the slide, and then allowed to dry.

By this method Brucella and the nuclei of the phagocytes stain deep red. The cytoplasm of the latter is a light pink; granules are not stained. Red blood cells stain weakly. Phagocytized bacteria appearing against the lightly stained cytoplasm are easily counted.

This method has also proved satisfactory for phagocytosis studies with streptococci, staphylococci, pneumococci, *H. influenzae*, and *H. pertussis*.

I wish to acknowledge with gratitude the advice of Dr. John A. Kolmer who suggested the use of carbolfuchsin.

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THE STANDARDIZATION OF CERTAIN FACTORS IN THE CUTANEOUS "VENOSTASIS" BLEEDING TIME TECHNIQUE*

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UNTIL all the factors concerned in hemostasis are known and can be tested individually by adequately quantitative methods, the determination of bleeding time will remain a necessary and valuable test of the "bleeding tendency." A disturbance of hemostasis obviously involves defects in either the process of coagulation, or the capillaries, or a combination of both. The presence or absence of a "bleeding tendency" would not necessarily be revealed even if one were to test for a coagulation defect by adequate methods and for capillary fragility by the suction or tourniquet technique. It is possible that a defect in coagulation might be neutralized by a compensatory response in the capillaries or vice versa. In other situations, however, the imperfections of one might augment those of the other. It is desirable, therefore, both for physiologic experimentation and for clinical purposes, to have a reliable method for the determination of bleeding time in order to study the end result of the relationships between the defects in coagulation and in the capillaries or peripheral tissues.

For this reason, we have undertaken a study of the reliability and limitations of the venostasis bleeding time test.[1, 2] This test was selected because venostasis usually prolongs the bleeding time of normal subjects[1] and hence tends to accentuate differences and defects that otherwise might be missed.

The tests reported in this study were performed exclusively on the bleeding tendency of the skin. It should be remembered that the results are not necessarily applicable to the mucous membranes and other tissues.[3]

EXPERIMENTAL

The Lancet.—The Sharpe and Smith lancet was used. It was selected because we believed the wide base might serve to distribute the pressure more equally. In a preliminary study it was found that setting the blade length at 2.5 mm. below the level of the *rim of the base* resulted in too great a percentage incidence of "no bleeding." The use of a 3.0 mm. blade length decreased but did not abolish the number of "no or zero bleeding" cuts.

Another difficulty was the sharpness of the blade. Cuts made with a sharp blade bleed more freely than those made with a dull blade. The blades were kept sharp with very fine pumice and a leather strap. If sharpened too frequently, the metal is worn away so that the thicker portion of the blade remains.

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New lancets in a single shipment were found to be well standardized as to width, but not as to thickness. Ideally, thin and replaceable blades, as in the Tocantins' instrument,[3] should be employed.

Control of Pressure.—Standardization of the depth of the puncture is one of the most essential features of a good bleeding time test. It seemed probable that control of the pressure placed upon the lancet before the blade is released might contribute to the uniformity of the punctures.

In order to control pressure, the lancet was mounted on a framework to which an arm rest was attached. The sliding bar which held the lancet was so weighted that when the lancet was placed on the average arm, its rim depressed the skin approximately 1 cm. This amount of pressure gave exceptionally short bleeding times in subjects with a well-developed pronator muscle and little subcutaneous fat. In these cases the skin was not depressed 1 cm. by the weighted lancet, and it became evident that a constant pressure did not give the desired uniformity of the punctures. We, therefore, devised a gauge for the lancet that would indicate when the skin was depressed exactly 1 cm., and when necessary manual pressure was added till that depth was reached. This procedure resulted in unduly long bleeding times in the very muscular subjects. It is obvious, then, that neither the application of the same amount of pressure in all cases nor the identical depression of the peripheral tissues was able to insure punctures of equal depth.

With the mechanical refinements used, the results showed a slight decrease in variability as compared with those obtained using the ordinary clinical procedure. However, the improvement was within the experimental error and hence was not significant. From this we conclude that with experience one can acquire a judgment in regard to pressure that is about as accurate as these mechanical devices for the production of uniform punctures.

The area of skin in which the punctures were made. With the subject seated and his forearm (left) in the supine position, the area over the pronator (lateral aspect of forearm below the cubital fossa) was washed gently with 95 per cent alcohol. (We have found that either forearm may be used.) The lancet was sterilized with alcohol and dried. After any hyperemia that may have occurred from the alcohol wash had disappeared, the pressure in a sphygmomanometer cuff about the arm was raised to 40 mm. Hg. A hemostat was used to prevent leakage of air from the cuff through the inflation bulb. The puncture or punctures were then made.

Since we were interested in comparing bleeding times in the same individual, it was necessary to make a number of punctures. Five series of two punctures were made on our 88 subjects as follows: with the pressure in the cuff at 40 mm. Hg, a puncture was made, and then five seconds later a second puncture was made, not less than one inch from the first puncture. The blood collected at each puncture was blotted with filter paper at ten-second intervals, timed with a stop watch. The filter paper was not pressed so firmly as to open the wound. The bleeding time is easily determined by multiplying the number of drops of blood on the filter by ten seconds.

After bleeding from the two punctures had ceased, the cuff was deflated and the arm was very mildly exercised. Then two more punctures were made an inch apart. This procedure was repeated until the bleeding time of five sets of two punctures each had been determined. This provided the data for analysis.

Subjects.—Eighty-eight subjects, 58 men and 30 women, of medical school age were used. Retests were made on 31 subjects, some of them being tested on several occasions.

Three additional subjects were excluded from this study because their mean bleeding times were definitely abnormal (vide infra). For some unknown reason an occasional subject continued to ooze blood-tinged serum for a relatively prolonged period. Data from such subjects were not used.

RESULTS AND DISCUSSION

The "clinically normal" maximum venostasis bleeding time from a single puncture. In Fig. 1 the 1,280 readings on the 88 subjects are distributed according to frequency. The bleeding times ranged from 0 to 310 seconds. The upper limit for "the clinically normal" found by Ivy, Shapiro, and Melnick[1] was 240 seconds. According to our results, 240 seconds includes 99.54 per cent of the readings. It is our belief that the few bleeding times above 240 seconds were due to striking a hidden vein because they were not typical of the other bleeding time measures obtained from the same individuals. Thus, 240 seconds as a "clinically normal" maximum venostasis bleeding time for a "one puncture test" is confirmed for the age group studied, unless a vein is accidentally punctured.

In setting a "clinically normal" maximal "venostasis" bleeding time at 240 seconds on the basis of a "one puncture test," patients with a mild to moderate "bleeding tendency" may be diagnosed as normal. This is likely because of the errors inherent in any relatively crude method. However, the errors usually tend to cancel each other when a number of tests are made. Hence the mean of several bleeding times more closely represents the true status.

The "clinically normal" mean or average venostasis bleeding time. In Fig. 1 the mean bleeding times of the 88 subjects are distributed according to frequency. The mean bleeding time of the subject who had the best cutaneous hemostatic mechanism by this test was twenty seconds, and that with the poorest was 125 seconds. Thus, a patient would be "clinically abnormal" if the mean bleeding time as determined by the test outlined above was significantly greater than 125 seconds.

COMMENT

From the preceding discussion of the results the advantage of making more than one test of bleeding time is obvious. However, the patient and the time factor have to be considered. The question then arises: What is the least number of punctures required to yield a reasonably reliable mean cutaneous venostasis bleeding time? A subsidiary question is: When two punctures are made, one within five seconds of the other, does the making of the first puncture modify significantly the bleeding time of the second puncture?

What is the least number of determinations required to yield a reasonably reliable cutaneous "venostasis" bleeding time? In Table I are listed the various

means and medians that may be used as a bleeding score. Medians were proposed to eliminate extreme readings which weight the means.

As can be seen from Fig. 1 the frequency of zero bleeding times is greater than that of any other bleeding time. Table II gives the distribution of the zero readings over the ten successive punctures. Due to the influence the zeros would have on the variability of the means or medians, these were recalculated using the first 3, 4, or 6 individual punctures that bled instead of the first 3, 4, or 6 punctures made (Table I).

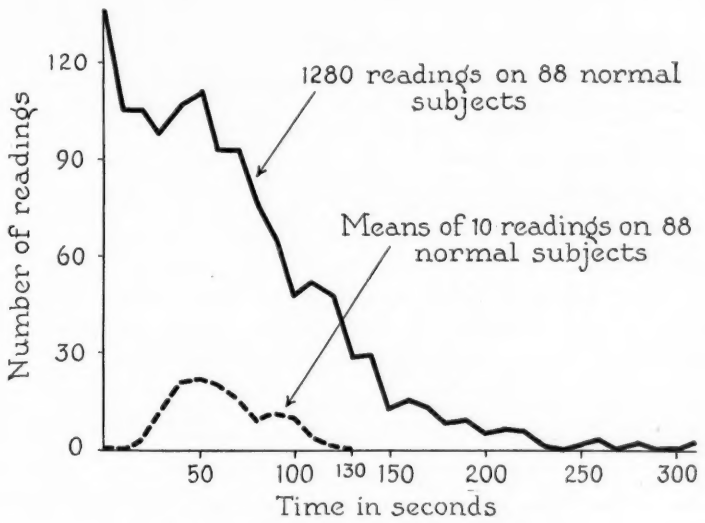

Fig. 1.—Distributions of bleeding times.
A = 1,280 readings on 88 normal subjects.
B = Means of 10 readings on 88 normal subjects.
Abscissae = Time in seconds.
Ordinates = Number of readings.

Regarding each of the listed means and medians in Table I as the result of a proposed bleeding time test, four criteria will be used to examine their relative value: (1) their similarity to the mean of 10; (2) their reliability as judged by the test-retest method; (3) their reliability as judged by the deviations from their means; and (4) the practical consideration which calls for the shortest test possible.

The deviations of each measure from the mean of 10 are shown in Table I, and the significance of the differences is indicated by the critical ratios. The mean of the first three odd punctures that bled is almost exactly the same as the mean of 10, and the median of the first four that bled shows only a slight difference. Since a critical ratio must be 2.60 or more to indicate a statistically significant difference with this number of cases,[4] it may be seen that the mean of the first 6 that bled, and the mean of the middle 6 are significantly larger than the mean of 10. The mean and the median of the first 4 made, and also the median of the first 3 made are significantly smaller than the mean of 10. The directions of these deviations suggest that the exclusion or preponderance of zero scores is responsible for the extent of variation.

The correlation of each of these measures with the mean of 10 is another demonstration of similarity. All the correlations are spuriously high because the individual readings from which each of the smaller means or medians is derived are also included in calculating the mean of 10. However, the correlations will not misrepresent the facts if the relationships are interpreted as showing the similarity of 128 bleeding time scores computed by each of two methods, and not as indicating the accuracy with which, knowing the mean of 10, one could predict the compared mean or median that would be obtained if the necessary six, four, or three additional punctures were made.

TABLE I

MEASURES OF BLEEDING TIME DERIVED FROM VARIOUS NUMBERS OF PUNCTURES. THE COMPARISON OF EACH WITH THE MEAN OF 10 PUNCTURES, AND THE SELF-CORRELATION OF EACH AFTER A RETEST

MEASURE OF BLEEDING TIME	TIME IN SECONDS	CORRELATION WITH MEAN OF 10	DIFFERENCE FROM MEAN OF 10	C.R.* OF DIFF. FROM MEAN OF 10	CORRELATION TEST-RETEST
Mean of 10 punctures	61.56 ± 2.1				+0.30
Mean of first 6 made	59.69 ± 2.3	+0.90	1.87	1.85	+0.34
Mean of first 6 that bled	66.95 ± 2.3	+0.87	5.39	4.73	+0.13
Mean of first 4 made	56.64 ± 2.7	+0.74	4.92	2.68	+0.30
Mean of first 4 that bled	65.23 ± 2.5	+0.77	3.67	2.25	+0.17
Median of first 4 made	53.28 ± 2.8	+0.74	8.28	4.40	+0.36
Median of first 4 that bled	62.19 ± 2.6	+0.73	0.63	0.35	+0.36
Median of first 3 made	48.28 ± 2.6	+0.69	13.28	6.95	+0.55
Median of first 3 that bled	58.13 ± 2.5	+0.65	3.43	1.72	+0.29
Mean of middle 6 made	64.06 ± 2.6	+0.94	2.50	2.61	+0.29
Mean of first 3 odd that bled	61.41 ± 2.4	+0.91	0.15	0.15	+0.33

*C.R. = Critical ratio = $\frac{\text{Obtained difference}}{\text{Sigma of difference}}$

Sigma difference = $\sqrt{(\sigma m_1)^2 + (\sigma m_2)^2 - 2r\sigma m_1\sigma m_2}$

r = Coefficient of correlation

Obviously, each addition of identical data raises a correlation. Therefore, the means of 6 would be expected to show the highest correlation in this particular situation. *It is thus of great interest to note that the mean of the first 3 odd punctures that bled has a correlation as high as the means whose correlations involved twice as many identical readings.*

The reliability of a test shows the degree of consistency with which it measures. One indication of reliability is a high correlation between two sets of results when, under controlled conditions, the test is repeated on the same individuals. Unfortunately, this "test-retest" information is available for only 31 cases. The correlations are given in Table I; they are all unduly low because the narrow limits of the data included in this study do not permit the range of variability between one individual and another which is necessary for any test to demonstrate its maximum value. Therefore, the significance of the "test-retest" correlations is for comparative purposes only. It will be noted that the zero scores are again a determining factor, since the highest correlation is the median of the first 3 punctures made, where the incidence of zero readings predominates, and the correlations of the means of data from which the zeros are omitted, are conspicuously inferior.

There was no consistent trend toward a longer or shorter bleeding time when the test was repeated on 31 subjects. The mean bleeding times of the 31 first tests were about the same as the means of the 31 repeat tests. The means or medians of 10, 6, 4, or 3 punctures for the 31 cases varied from 50 to 65 seconds in both the first and repeat tests. It was apparently mere chance as to which was the higher, each one being slightly superior as gauged by about half of the measures. As indicated by the low correlations (Table I) there was little consistency between the first and repeat tests of each individual. This is to be expected with such a homogeneous group.

TABLE II

INCIDENCE OF ZERO READINGS

CONSECUTIVE PUNCTURES	1	2	3	4	5	6	7	8	9	10
% of total (128 cases)	20	19	9	9	11	10	8	8	6	5
% of men (58 cases)	21	16	10	8	7	16	12	8	5	7
% of women (30 cases)	19	21	9	10	15	5	5	7	8	5

Another criterion of reliability is a small standard error or deviation of the mean of a series of tests. The standard error, or sigma, of a mean (σm) is a measure of variability which indicates the range within which a "true mean," or the mean of an infinite number of cases, would have a 67 per cent chance of falling. It is also the range within which 67 per cent of the means would fall if other sample groups of the same size were tested under conditions identical to those producing the original mean. Therefore, if the range designated by the sigma of a mean is small, the test is consistent.

The standard errors of the means and medians listed in Table I were obtained by assigning to each person a bleeding time based on the mean of 10 punctures or the median of 3, etc., and finding the sigmas of the mean of the 128 bleeding times so defined. They are not strikingly dissimilar. It may, therefore, be concluded that if one wishes to determine the average bleeding time of a group of about a hundred people, any of the proposed tests would offer approximately the same degree of reliability. However, there would be a trend in the direction of greater consistency in the measures whose individual scores were based on a larger number of punctures. An exception to this trend is noted in favor of the mean of the first 3 odd punctures that bled.

This material on standard errors of the means or medians of 128 cases gives no indication of the reliability of the various means or medians for determining the bleeding time of individual persons, which is of much greater clinical importance than the measurement of groups. *The standard error of a mean or median of the punctures made on any subject is the range within which the chances are 2 to 1 that his "true bleeding time" would lie if established by an infinite number of punctures.* Naturally this range differs in individuals. By finding the average standard error of the 128 means and medians, a comparison can be made of their reliability as individual bleeding time tests. The mean standard errors are listed in Table III, together with their correlations and differences from the average sigma of the means of 10.

The formula for deriving a sigma of the mean being

$$\frac{\text{Sigma of distribution}}{\sqrt{\text{Number of cases}-2}}$$

when there are 10 cases, and

$$\frac{\text{Sigma of distribution}}{\sqrt{\text{Number of cases}-3}}$$

when there are less than 10, it can be seen that the measures based on fewer than 10 punctures were heavily penalized.[5] It may be argued that such an inequitable comparison is of little value, but this is not the case, since the issue will be encountered every time one investigates the significance of a difference between bleeding measures made under varied conditions, as, for example, before and after treatment.

TABLE III

RELIABILITY OF THE MEASURES AS GAUGED BY INTERNAL CONSISTENCY

MEASURE OF BLEEDING TIME	MEAN SIGMA OF MEANS	DIFFERENCE FROM SIGMA MEAN OF 10	C. R.	CORRELATION WITH SIGMA MEAN OF 10
Mean of all 10	14.73 ± 0.51			
Mean of first 6 made	22.51 ± 0.92	7.78	14.88	0.80
Mean of first 6 that bled	17.15 ± 0.86	2.42	5.69	0.96
Mean of first 4 made	31.09 ± 1.54	18.61	12.48	0.57
Mean of first 4 that bled	29.57 ± 1.53	14.84	13.86	0.60
Median of first 4 made	33.40 ± 1.60	18.67	13.24	0.51
Median of first 4 that bled	31.72 ± 1.60	16.99	12.67	0.62
Median of first 3 made	31.33 ± 1.94	16.60	9.70	0.56
Median of first 3 that bled	29.02 ± 1.84	14.29	8.82	0.53
Mean of first 3 odd that bled	25.31 ± 1.71	10.58	6.82	0.45

The differences between the mean sigmas of the means of 10 and the mean sigmas of all the other measures are statistically significant. The large critical ratios are due to the spuriously high correlations (see footnote to Table I). It can be seen by inspection of the table that except for the sigmas of the means of six punctures, the differences between any two of the other measures would not be significant unless equally high correlations exist between them. The sigmas of the means of the 3 odd punctures might also be an exception. However, statistical analyses were not made to confirm this.

The standard errors of the means and medians of individual cases are open to criticism from a statistical standpoint because the formulas from which they are calculated are based on the assumption that the data fall into a "normal distribution curve." The curve obtained by plotting all the raw scores, as shown in Fig. 1, epitomizes the skewed frequency which was typical of nearly all the individual curves. Probably the most important factor in causing this marked positive skew was the length of the lancet blade. Fig. 1, showing the curve of the means of 10 punctures, illustrates the fairly normal distribution characteristic also of the other means and medians.

To eliminate all errors arising from the asymmetrical distribution, the data in Fig. 1 were also analyzed in terms of per cent, using the means of 10 as the point of reference. The 128 cases were divided into two groups, those having a mean bleeding time (mean of 10 punctures) of one minute or less and those

having a mean bleeding time between one and two minutes. The per cent of these groups with a bleeding time of one, two, or three minutes on the basis of the other means and medians is shown in Table IV.

TABLE IV

COMPARISON OF SHORTER TESTS WITH THE MEAN BLEEDING TIME OF 10 PUNCTURES

MEASURE OF BLEEDING TIME	A % OF PERSONS WHOSE BLEEDING TIME IS 0-60" (MEAN OF 10 PUNCTURES) HAVING B. T. 1', 2', OR 3'			SCORE*	B % OF PERSONS WHOSE BLEEDING IS 61-120" (MEAN OF 10 PUNCTURES) HAVING B. T. 1', 2', OR 3'		
	1'	2'	3'		1'	2'	3'
Mean of first 6 made	91	9	0	169	16	78	6
Mean of first 6 that bled	78	22	0	160	10	82	8
Mean of first 4 made	86	14	0	150	32	64	4
Mean of first 4 that bled	76	24	0	150	20	74	6
Median of first 4 made	88	12	0	144	36	56	8
Median of first 4 that bled	79	21	0	145	26	66	8
Median of first 3 made	93	7	0	145	44	52	4
Median of first 3 that bled	85	15	0	147	32	62	6
Mean of first 3 odd that bled	86	14	0	152	31	66	3

*This score is the sum of the per cent values in column 1A and column 2B. It is an arbitrary measure for evaluation.

The measures which included zero readings show the greatest per cent of agreement with the group which had a bleeding time of one minute or less (on the basis of the mean of 10), and the measures excluding the zeros were in better agreement with the group having a bleeding time between one and two minutes. By summing the per cent values listed for each test in column one (1') of the first group with those of column two (2') of the second group, an arbitrary score was devised by which to evaluate the tests. As was pointed out in the discussion of the correlations of the means, the inclusion of the same raw data in computing the two measures, gives an advantage to those based on the largest number of punctures. *As in the previous case an exception appears in favor of the mean of the first three odd punctures that bled.*

TABLE V

BLEEDING TIME AVERAGES OF THE SUCCESSIVE PUNCTURES

SUBJECTS	1	2	3	4	5	6	7	8	9	10	TOTAL
All cases	47.4	53.7	55.6	64.9	53.0	76.9	59.4	67.9	62.5	71.2	61.5
All cases—zeros excluded	59.5	66.1	61.4	72.3	59.6	85.6	64.6	73.6	66.6	75.3	68.4
Men only	43.4	56.7	60.6	63.3	55.9	77.0	62.0	70.8	70.6	72.5	63.2
Women only	48.4	50.9	51.0	67.6	50.4	76.7	57.0	65.2	55.1	70.0	59.2

The fourth consideration in selecting one of the proposed bleeding time measures is the obvious advantage of brevity. The assumption that the test would be improved by increasing the number of punctures was invalid in this case. As is shown in Table V there is a decided trend toward a longer bleeding time as the test proceeded. This is also shown by the fact that the mean of the first 5 punctures (128 cases) is 54.38, and the mean of the last 5 is 68.59. The

difference between the two means is statistically significant, since the critical ratio is 5.24. *A progressive hyperemia seems to be the most pertinent interpretation of this cumulative error.*

Because the first two punctures included more than a third of the zero scores, and the last two were unduly prolonged, the means of the middle six punctures were computed (see Table I) and used as a standard in place of the means of 10. The substituted values did not gain sufficiently in uniformity to offset the statistical disadvantage of omitting two-fifths of the readings. This standard was, therefore, less satisfactory than the means of 10.

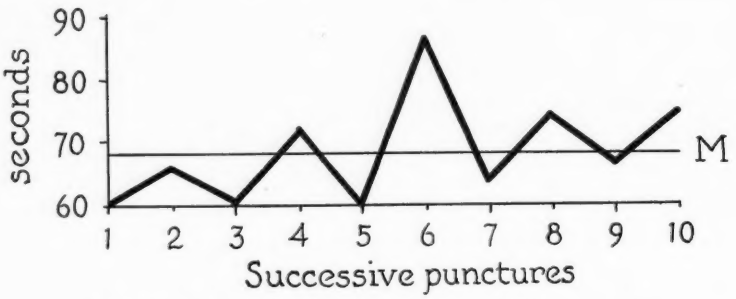

Fig. 2.—Average bleeding times of successive punctures.
Abscissae = Successive punctures.
Ordinates = Mean bleeding time in seconds. Zero readings excluded.
M = Mean of all punctures. Zero readings excluded.

Does a first puncture modify the bleeding of a second puncture made five seconds later? Table V and Fig. 2 also reveal another source of error, where one puncture follows another in five seconds. Apparently the first puncture of each pair was sufficiently traumatic to cause a transitory hyperemia or change, probably due to an axone reflex. The only exception to the sharp alternations is puncture 2, when the zero readings are included. When all the first punctures, the odd tests, are compared with the second, or even tests, there is a significant difference between them. The mean of the odds is 56.88, the mean of the evens is 68.12, and the critical ratio is 5.70.

It is very interesting that in spite of the marked difference between them, the odd and even punctures have a correlation coefficient of +0.63. A correlation of this type constitutes the "split-half" technique for determining the reliability of a test. Considering the similarity of the data, this is a fairly high correlation, certainly far superior to the "test-retest" relationship. The fact that a correlation of single punctures made within five seconds of each other is twice as high as the correlation of the means of 10 punctures made on different days may be interpreted in two ways. It may mean that the control of all variables, except the site of puncture, as was the case with the simultaneous readings, minimized certain physiologic fluctuations; on the other hand, it may simply indicate that sufficient errors arise in the long test to impair its reliability.

The reason for making the readings in pairs was to save time. *Analysis of the results shows clearly that more uniform results are to be expected from making the punctures one at a time.*

Inspection of Fig. 2 suggests the use of the first three odd punctures that bled as the most consistent test of bleeding time. This measure compared favor-

ably with the others: (1) the 128 means thus obtained were almost identical to those which resulted from averaging 10 punctures; (2) reliability judged by the "test-retest" method was about average; (3) reliability based on deviations was relatively high; and (4) a three-puncture test is undeniably short. It seems, therefore, to fulfill all the requirements stipulated for the evaluation of the proposed bleeding time measures.

Recommendation.—Using the lancet we employed, it is recommended that a relatively reliable mean venostasis bleeding time may be determined as follows: (1) The pressure in the cuff is elevated to 40 mm. Hg. The puncture is made. If bleeding occurs, the bleeding time is recorded; if it does not, nothing is recorded. The cuff is decompressed. (2) Five minutes later the cuff is inflated again, and the puncture is made. If bleeding occurs, the bleeding time is recorded; if it does not, nothing is recorded. This procedure is repeated until three bleeding times have been recorded. The mean bleeding time is the average of the three bleeding times. In our 88 subjects the mean bleeding time of three punctures was 61.41 ± 2.43, as compared to the mean of 10 punctures which was 61.56 ± 2.08. It should, however, be recalled that our three punctures were the first punctures of each of three pairs and were not made singly as recommended above.

SUMMARY

Mechanical refinements directed toward increasing the accuracy of the use of a spring, flat-bladed lancet in the hands of an operator of some experience did not prove to be of significant value. The blade of the lancet was set at 3 mm. Venostasis of the forearm was produced by placing a sphygmomanometer cuff about the arm and inflating it to 40 mm. Hg pressure. The skin over the pronator was punctured with the lancet. The blood was gently blotted at ten-second intervals until bleeding ceased.

A study of the reliability of the venostasis bleeding time technique was made on 88 "normal" subjects by a multiple skin puncture method. Five pairs of punctures of the skin were made on each, the second puncture of the pair being made five seconds after the first. The cuff was deflated and the arm was moved about to restore circulation between each pair of tests. The subjects included 58 men and 30 women from about 21 to 40 years of age.

A "clinically normal" *maximum* venostasis bleeding time of 240 seconds for a "one puncture test" was confirmed. A "clinically normal" *maximum mean* venostasis bleeding time of 125 seconds was found on making 10 punctures in each of the 88 subjects. A "clinically normal" *mean* venostasis bleeding time of 61.56 ± 2.08 seconds was obtained for this group of 88 subjects. No significant sex difference in the mean bleeding time was found. The results on 31 subjects who were retested showed no significant variation in the mean bleeding time.

Statistical analysis of the results showed (a) that the second puncture of each pair bled significantly longer on the average than the first, (b) that the mean bleeding time of the last 5 punctures was significantly greater than the first 5, and (c) that a relatively reliable mean bleeding time for one setting may be

obtained by making one puncture during each inflation of the cuff, and averaging the time of the first three that bleed.

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CHEMICAL

HEMOGLOBIN ESTIMATION WITH UNDILUTED REDUCED BLOOD*

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SAHLI[1] in 1902 first used the color intensity of acid hematin as a basis for the clinical estimation of hemoglobin. The original Sahli instrument has seen many modifications and reconstructions, e.g., the Autenrieth-Königsberger hemometer,[2] the Newcomer hemometer,[3] and the Haden-Hausser instrument[4] have been introduced. In Europe there has been constructed in recent years the Zeiss-Ikon hemometer[5] in addition. All these models, like the original Sahli instrument, use the brown color of acid hematin which has to be compared with a standard color (fluid or glass). This "Sahli principle" is probably the one most commonly used for hemoglobin estimation in routine work. It is well known that the brown color which develops from addition of acid to the blood depends on various reactions. They in turn are supposed, as a whole, to depend on the hemoglobin content and hence, if standard conditions are used, to lead to a color intensity which is proportional to the hemoglobin previously present in the blood.

Newcomer[3] in 1930 published a very profound investigation about the optical behavior of acid hematin. His experience suggested that the curve of color development against time had a rather regular slope. The question remained whether the blood solution after being acidified really "acts for the present purpose as if it formed a true solution" (Newcomer[3]). In a series of publications[6-11] during the last few years, I was able to demonstrate that the colloidal character of acid hematin solutions can very easily be evidenced by the influence upon the hemometric readings of different experimental conditions. In experiments with human blood I could show deviations as high as 14 per cent in the hematin color intensity as compared with the photometrically determined oxyhemoglobin value. I wrote (1937) with reference to the colorimetry of the hematin color, "the latter is a term of very doubtful value. It should disappear from the clinic as a measure of the hemoglobin content."[10] Heilmeyer and von Mutius[12] (1938), in comparing the hematin method with other more reliable methods of hemoglobin determination, also found an inaccuracy up to 11 per cent and so did Humperdinck.[13] Heilmeyer and von Mutius state "that the method should be eliminated as far as possible for scientific purposes"[12]; they are of the opinion that the procedure might still be satisfactory for medical practice because of the lack of any other reliable and handy method.

In 1937[9] I pointed out that the colorimetry of reduced hemoglobin, introduced by Bürker,[14] and in connection with the use of the Pulfrich photometer recommended by Heilmeyer,[15] could well be simplified with a handy instrument

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for routine work. In 1938 I learned that such an instrument had been proposed independently by the Danish physician Dr. M. Philipsen and was just brought on the market.[16] After having carried out comparative investigations with the new instrument (Sicca hemometer*), a brief report in connection with my previous publications in that field may be justified.

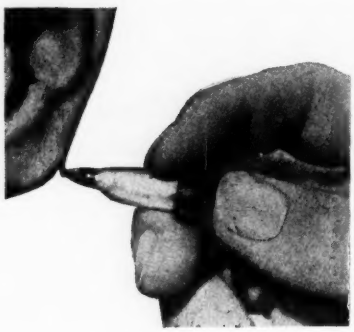

Fig. 1*A*.

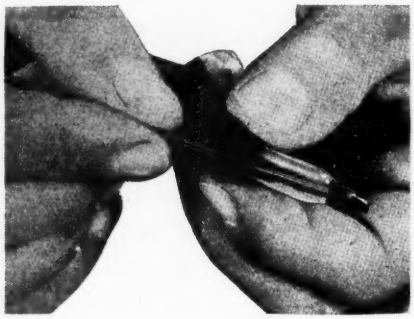

Fig. 1*B*.

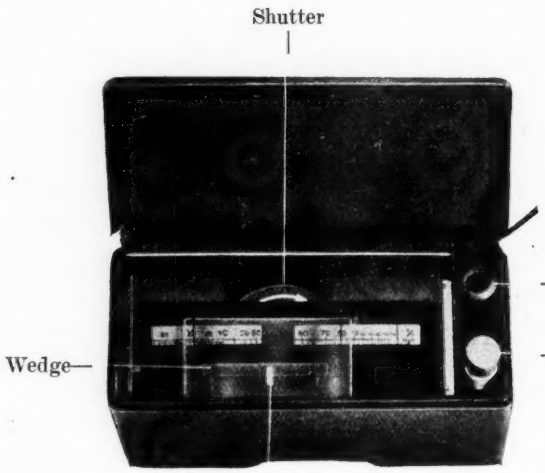

Fig. 2.

SICCA HEMOMETER

One of the advantages of the new method is that the blood need not be measured but simply taken in the hand pipette (Fig. 1*A*). Without being diluted it is reduced, hemolyzed, and stabilized by addition of a trace of a reagent powder containing sodium hydrosulfite, saponin, oxalate, and sodium chloride (Fig. 1*B*).

The main part of the hemoglobinometer is the wedge-shaped chamber. It is formed by a glass base plate and a covering glass piece ground to form a wedge-shaped hollow. The reduced blood specimen in the hand pipette is directed into the wedge-shaped chamber, thus forming a blood color wedge.

*Manufactured by the Testa-Laboratorium, Vedbaek, Denmark, U. S. A. Pat. No. 2,163,467.

The wedge chamber lies on the scale plate which has a window showing the standard color. Both blood and standard are illuminated from beneath by an electric lamp, the light passing some particularly selected filters. Fig. 2 shows the pattern of the apparatus. For determining the hemoglobin content the chamber is moved along until the specimen color corresponds to the standard. A marked line on the glass base plate indicates the hemoglobin value on the scale. The instrument is adapted to two ranges of hemoglobin content. This involves the use of two color standards, one of which is kept covered by a shutter. The standard most appropriate is uncovered by turning the wheel marked with an arrow. The arrow automatically points to that side of the scale to be read. An additional wedge ground to double height and marked "2" may conveniently be used for low hemoglobin values. When using this wedge all readings have to be divided by two.

TABLE I

STANDARDIZATION OF SICCA HEMOMETER WITH PHOTOELECTRIC OXYHEMOGLOBIN DETERMINATIONS

(Comparison With Hemoglobin Values Obtained From Blood Iron)

NUMBER	SICCA READINGS (MEAN OF 5-10) SCALE PARTS	HEMOGLOBIN (FROM SICCA) GM./100 C.C.	$O_2HB.$ (PHOTOELECTR. EVELYN) GM./100 C.C.	HEMOGLOBIN (FROM BLOOD IRON WONG-WALKER) GM./100 C.C.
1	87.0	12.7	12.8	13.4
2	74.3	10.9	11.5	11.3
3	101.6	14.9	15.2	15.2
4	87.0	12.7	12.1	12.8
5	104.0	15.2	14.8	14.8
6	65.0	9.5	9.4	9.5
7	102.7	15.0	15.1	15.1
8	81.4	11.9	12.4	11.9
9	103.5	15.1	14.5	14.2
10	100.0	14.6	14.0	13.7
11	97.9	14.3	14.0	14.9
12	100.8	14.8	14.6	14.9
13	81.7	12.0	12.1	11.6
14	94.3	13.8	14.3	13.7
15	98.6	14.4	14.2	13.7
16	88.9	13.0	13.5	13.2
17	75.1	11.0	10.4	10.7
18	85.9	12.6	12.5	13.1
19	111.7	16.4	16.7	16.4
20	87.9	12.9	13.7	13.1
21	100.9	14.8	15.3	14.0
22	91.2	13.3	12.7	13.1
23	90.0	13.2	13.2	13.2
24	82.3	12.0	12.1	12.5
25	108.1	15.8	16.0	16.1
Average	92.07		13.48	13.44

Therefore 100 scale parts Sicca = 14.64 grams of hemoglobin per 100 c.c.

STANDARDIZATION

Because of a mistake in shipping, I had to build in a new set of filters in the instrument I used first. Thus a restandardization of the hemometer became necessary. Twenty-five samples were taken from different patients and protected against coagulation by adding some oxalate or liquoid "Roche." The hemoglobin in each sample was determined electrophotometrically according to Evelyn.[17] In addition the iron content of the blood was determined following

Walker's[18] modification of Wong's method. The hemoglobin was calculated on the basis of 0.336 per cent iron. The same 25 samples were also used for hemoglobin determinations in the Sicca hemometer. The results of five to ten readings with each sample were averaged. Table I contains the synopsis of the results.

The average of the readings on the scale in the 25 blood samples was 92.07. The average for the oxyhemoglobin content photoelectrometrically measured, according to Evelyn, was 13.48 Gm. per 100 c.c. From these two figures was calculated that 100 parts on the scale of this particular instrument (A) equal 14.64 Gm. of hemoglobin per 100 c.c. Using this correlation, the hemoglobin content for each of the 25 samples was calculated from the respective Sicca readings. The results are shown in Table I and can be compared with the respective figures from the photoelectrical readings and the iron determinations.

TABLE II

COMPARISON BETWEEN TWO SICCA HEMOMETERS (A AND B)

A Standardized by the Author; B Standardized by the Manufacturers

NO.	GRAMS HB. PER 100 C.C.		NO.	GRAMS HB. PER 100 C.C.	
	A	B		A	B
1	12.5	12.4	13	14.3	14.0
2	12.6	12.8	14	12.9	13.2
3	14.6	15.0	15	11.9	11.7
4	14.9	15.2	16	11.9	12.3
5	8.7	8.7	17	13.3	13.4
6	12.3	12.2	18	13.1	13.1
7	13.1	13.1	19	12.6	12.4
8	12.7	12.7	20	12.0	11.9
9	13.3	13.5	21	13.2	13.2
10	12.4	12.5	22	13.9	14.2
11	15.6	16.2	23	13.8	13.6
12	12.8	12.9	24	14.5	14.4
			25	14.6	14.4

TESTING OF MANUFACTURERS' STANDARDIZATION

The second Sicca hemometer (B) was standardized by the manufacturers in Denmark in the manner commonly used there (in cooperation with Professor A. Krogh's Zoofysiologisk Laboratorium) on the basis of 100 parts of the scale equal 18.5 volume per cent of oxygen (Haldane); 18.5 volumes per cent of oxygen correspond to 13.85 Gm. of hemoglobin per 100 c.c. Thus the latter is at the same time the standardized hemoglobin value for 100 parts on the scale. In other words, one part on the scale means a little less hemoglobin with the instrument *B* than with the instrument *A*. A total of 25 other human blood samples were read in the two Sicca hemometers. The same wedge chamber filled with the prepared blood was placed first in one instrument, and after the readings were taken (usually 5 to 10) placed in the other one. The order of the use of the two hemometers was alternating, thus also controlling any influence of time. The readings for each instrument were multiplied by their respective factors (0.1464 for A and 0.1385 for B). Table II shows the results obtained.

The hemoglobin content of the samples varied from about 8.7 to about 16.0 grams per cubic centimeter. The average of the values for the 25 blood samples

obtained with the two instruments is 13.1 and 13.2 Gm. per 100 c.c. In the individual results there is also a good agreement between the two instruments. The deviation is less than 1 per cent in 19 of the 25 cases, and never exceeds 2 per cent of the average reading.

The low dispersion for individual readings was also proved in the following manner: In 50 determinations with the Sicca hemometer in which ten readings had been taken for each sample, the average of the first three (R_3) and of the ten readings (R_{10}) was compared. In 48 of the 50 cases the per cent deviation of the two averages: $\frac{\pm (R_3 - R_{10})\ 100}{R_{10}}$ was not greater than 2; in the remaining 2 cases the deviation was 2.5 and 3.5 per cent, respectively. This means that by taking the average of three readings with the same specimen one can be certain of having a result correct for all practical purposes.

INTERCHANGEABILITY OF PARTS

One of the most important claims for the Sicca hemometer is that all spare parts are supposed to be interchangeable without restandardization. The parts of the wedge chamber evidently are the most vulnerable elements of the instrument in practical use. Three different base plates equipped with three different wedges were filled with blood from one sample. The readings were taken in the same instrument. The hemoglobin values obtained for the three wedge chambers were: 10.54; 10.64, and 10.45 Gm. per 100 c.c., respectively. This means a deviation of less than 1 per cent from the average.

Table III gives the average results of low hemoglobin values in four cases, two of them artificial dilutions, and two cases of pernicious anemia. The data obtained with wedge "2" in the Sicca are compared with those obtained with Evelyn's photoelectric method.

TABLE III

LOW HEMOGLOBIN VALUES DETERMINED WITH SICCA AND PHOTOELECTRICALLY

SAMPLE	SICCA HEMOMETER WEDGE "2" GM. HB./100 C.C.	PHOTOELECTRIC METHOD EVELYN GM. HB./100 C.C.
Diluted blood I + 0.9 per cent NaCl	5.33	5.41
Diluted blood II + 0.9 per cent NaCl	3.84	3.92
Pernicious anemia; Case P.	5.33	5.30
Pernicious anemia; Case C.	5.36	5.44

The photoelectric oxyhemoglobin determination (Evelyn) and the results obtained with the Sicca instrument, as a rule, were in very close agreement for the low as well as for the normal hemoglobin concentrations. In this connection it is of particular interest that, according to personal information by Dr. H. Hesse, a large series of hemoglobin estimations carried out in Montreal, Canada, with the Sicca instrument and with Van Slyke's method of determination the oxygen capacity never showed deviation exceeding 3 per cent.

SUMMARY

In my opinion some advantages of the new type of hemoglobinometer are:

(1) The use of undiluted blood eliminates the sources of error involved in diluting. The handling of the Sicca hemometer is very simple and needs no particular training.

(2) The use for colorimetry of acid hematin, repeatedly criticized in earlier work, is abandoned in favor of the color of reduced blood. The latter is stable and independent of time, the errors due to inadequate time for reading being eliminated.

(3) The use of constant glass standard and constant electric light in connection with correct filters allows observations with great optical contrast, eliminates the effects of voltage variations and plasma color (icteric blood), and decreases the personal equation to a minimum.

(4) The dispersion of repeated readings with the same or with different Sicca hemometers is small, i.e., the reproducibility is particularly good.

(5) Spare parts can be used without new standardization.

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THE MEASUREMENT OF CELL VOLUME OF BLOOD BY THE EVANS BLUE DYE METHOD*

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THE hematocrit method for the determination of the cell volume in blood, as developed by Wintrobe,[1] Guest,[2] and others, has been of great service in both physiologic and clinical studies. It has the advantage of being simple and direct, and further, it gives separate readings for erythrocytes and leucocytes. It requires approximately 1 c.c. of blood. Careful studies of this method by Ponder[3] and Hirota[4] have shown, however, that it is not without its difficulties. The values obtained at 3,000 r.p.m. for thirty minutes, which is the condition commonly used, are arbitrary values, but, Ponder states, are correct ±5 per cent. It is generally accepted that the error is within 2 or 3 per cent. Low speed and short duration of centrifugation result in a large volume of packed cells. Centrifugation at higher speeds or for longer times causes the volume of packed cells to become smaller to the point where fluid is squeezed out of the cells by centrifugal force. Moreover, various bloods do not attain the same equilibrium at the same time or speed of centrifugation.

A method for determination of cell volume, called the cell opacity method, has recently been described.[5] This method depends upon the obstruction to light, of 660 mμ, by a suspension of blood in citrate solution. The amount of light transmitted actuates a photoelectric cell, and the intensity of the current is measured by a galvanometer in a photoelectric colorimeter. An empirical correlation between this reading and the cell volume obtained by the hematocrit method is made. The method uses only 10 or 20 c. mm. of blood and requires only a few seconds to carry out. The values on relatively normal bloods agree within 2 or 3 per cent with those obtained by the hematocrit method.

To obviate the difficulties of the hematocrit method and to serve as a standard of reference, Ponder[6] suggested the use of a colorimetric method based upon the addition of a solution of hemoglobin in plasma to whole blood from the same individual. The hemoglobin concentration of the plasma is determined before and after the addition, and the volume of the cells is thus calculated. The technical difficulties of this method in our hands have been considerable. It is open to the further objection that about 35 c.c. of blood are required for each determination. Further, what Ponder calls a solution of hemoglobin really contains all the soluble parts of the cell, high in potassium and

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phosphate, obtained by freezing and lysis, and perhaps some cellular detritus. Practically, the effect of these substances on osmotic pressure is greatly reduced because the hemoglobin solution is diluted 1:15.

The method reported here is a colorimetric method which we think is free from these objections. The principle of the method is as follows: A known amount of blood is mixed with a given amount of the dye, Evans blue, also called T-1824. The concentration of the dye in the plasma is determined colorimetrically with a photoelectric colorimeter. Because the dye is contained wholly within the plasma, the volume of the plasma is determined by this measurement; from this the relative amount of the plasma in whole blood is calculated as per cent, and the cell volume is obtained by difference. The method is more laborious than the hematocrit method or the cell opacity method. It is offered primarily as a standard of reference rather than as a clinical method, although it may be so used.

PROCEDURE

Evans blue (Merck) is dried at 110° C. and made up into solution with distilled water so that it contains 400 to 800 mg. of dye per liter. One half to 1 c.c. of this solution (containing 400 μg. of dye) is placed in a test tube of 3 to 4 c.c. capacity and evaporated to dryness at 70° C.

A measured amount of blood obtained by venepuncture is placed in a tube coated with a calculated amount of dried oxalate (60 per cent ammonium oxalate and 40 per cent potassium oxalate) or heparin to give a final concentration of 0.2 per cent oxalate or 2.0 to 2.5 units of heparin per cubic centimeter of blood. An aliquot is centrifuged. One cubic centimeter of plasma is placed in a tube containing the dye as described above (or a smaller volume but in the same proportion to the dye). The tube is shaken and the dye readily goes into solution. Of the plasma containing dye 0.1 c.c. is removed and placed in 9.9 c.c. of physiologic saline solution (0.85 per cent of sodium chloride) in a tube standardized for use in the photoelectric colorimeter. (The saline prevents precipitation of protein which would occur if the plasma were diluted with water.) Of the dye-free plasma 0.1 c.c. is placed in a similar colorimeter tube containing 9.9 c.c. of saline solution to serve as a "blank." The pipettes to be used for this purpose are those described by Levy.[7] These pipettes are calibrated "to contain," and automatically deliver the required volume, because they are constricted at the upper mark to a hairline diameter. The sample of plasma is delivered into the pipette washed with the saline solution.

A suitable photoelectric colorimeter, such as the Evelyn or the Klett-Summerson colorimeter, is arranged for use with a filter transmitting light at approximately 620 mμ. This filter was selected as most suitable by Gibson and Evelyn[11] after a study of the absorption curve of the dye. The blank is inserted and the machine is adjusted to read at 100 on the Evelyn or at 0 on the Klett-Summerson colorimeter. The dye tube is then inserted and the reading made. The dye has a very intense color, and the optimal concentrations for accurate reading in the Evelyn or Klett-Summerson colorimeter are approximately 15 to 50 μg. of Evans blue in a volume of 10 c.c. By adding varying amounts of

saline or plasma to the dye different readings are obtained which establish a straight line, as shown in Fig. 1.

The procedure for analysis of blood is similar to the quantitation of the dye in plasma. One, 2, or 3 c.c. of oxalated or heparinized blood are placed in a tube containing 400 μg. of Evans blue. (If 1 c.c. of blood is used, either half the amount of dye or twice the amount of diluent should be used.) The tube is stoppered and shaken until the dye is completely dissolved. The tube is then centrifuged at moderate speed, 1,500 r.p.m., for fifteen minutes. An aliquot of 0.1 c.c. of the supernatant plasma is added to 9.9 c.c. of saline in a colorimeter tube. The same plasma blank as above is used to determine the point of zero concentration of dye. The concentration of dye in the sample analyzed is obtained from the galvanometer reading by the use of the curve in Fig. 1. The cell volume is then calculated as follows:

A = amount of dye used; μg.
Z = concentration of dye determined in supernatant plasma; μg. per cubic centimeter.
S = volume of blood used; c.c.
P.V. = plasma volume; per cent of whole blood.
C.V. = cell volume; per cent of whole blood.

$$\frac{A}{Z \times S} \times 100 = \text{P.V.}; \quad 100 - \text{P.V.} = \text{C.V.}$$

Example: A = 400; Z = 370; S = 2.

$$\frac{400}{370 \times 2} \times 100 = 54.0 = \text{P.V.}; \quad 100 - 54.0 = 46.0 = \text{C.V.}$$

It is obvious that this method gives a measure of the volume of plasma in a given amount of blood, and hence the cell volume obtained by subtraction includes all the fixed elements in the blood, and not in the erythrocytes alone.

The first step in applying this method is for the operator to determine that he can make adequate recovery of the dye in water or saline, and then in plasma, and thus establish his curve. This curve can be used without making a plasma determination for each blood analyzed so long as the same lot of dye is used.

No attempt was made to handle the blood without loss of carbon dioxide or exposure to the air, but it was treated with reasonable care. It was kept in tubes nearly filled and always stoppered, and was analyzed within one hour after venepuncture. All the glassware was carefully calibrated. It was washed in cleaning solution before each use, and then repeatedly washed with distilled water. If the precaution of using cleaning solution is not observed, the blue dye will adhere to the glass.

Five cubic centimeters of blood are adequate for this determination in duplicate; two 1.0 c.c. samples are measured into tubes containing dye, the remainder is centrifuged to obtain plasma for the blank and for the determination of the concentration of dye in the plasma to check against the curve. If the centrifuging is done in a hematocrit tube, the cell volume may be read before the plasma is removed, and thus a comparison made without extra blood being required.

EXPERIMENTAL RESULTS

We wish to compare the results of simultaneous determinations of cell volume on the same blood by the hematocrit method, the cell opacity method, and the colorimetric Evans blue method. We used 15 c.c. of blood to enable us to make observations on known concentrations of dye in plasma on each blood, and quadruplicate determinations of cell volume employing aliquots of 1, 2, or 3 c.c. of blood. The results of the 16 consecutive determinations most recently made are given in Table I.

TABLE I

IDENTIFICATION			CELL VOLUME				COMPARISON OF RESULTS	
			1	2	3	4	5	6
DATE	SUBJECT	ANTICOAGULANT	HEMATOCRIT METHOD R.B.C. (%)	CELL OPACITY METHOD (%)	HEMATOCRIT METHOD R.B.C. + BUFFY COAT* (%)	EVANS BLUE METHOD (%)	1-2 (VOL./100 VOL. BLOOD)	3-4 (VOL./100 VOL. BLOOD)
12/31	T. H.	Oxalate	49.0	46.0	49.5	47.5	+3.0	+2.0
1/ 2	A. T.	Oxalate	48.0	46.5	48.5	46.2	+1.5	+1.3
1/ 2	T. H.	Oxalate	47.0	47.5	47.5	45.4	-0.5	+2.1
1/ 3	A. T.	Oxalate	47.5	45.5	48.0	47.5	+2.0	+0.5
1/ 4	T. H.	Heparin	50.0	49.5	50.5	47.5	+0.5	+3.0
1/ 6	A. T.	Heparin	47.0	48.0	47.5	45.6	-1.0	+1.9
1/14	G.†	Oxalate	31.0	28.5	31.5	30.0	+1.5	+1.5
1/16	A. T.	Oxalate	49.2	49.2	49.7	48.0	0.0	+1.7
1/17	H. S.	Oxalate	47.0	47.5	47.5	45.0	-0.5	+2.5
1/17	E. M.	Oxalate	41.7	41.4	42.2	41.0	+0.3	+1.2
1/23	T. H.	Oxalate	49.5	47.5	50.0	47.0	+2.0	+3.0
1/23	F. R.	Oxalate	46.0	45.5	46.5	44.3	+0.5	+2.2
1/24	M. C.	Oxalate	45.0	44.5	45.5	42.9	+0.5	+2.6
1/24	C. C.	Oxalate	46.0	45.5	46.5	44.5	+0.5	+2.0
1/27	J. Mc.	Oxalate	44.7	45.0	45.2	42.7	-0.3	+2.5
1/27	A. T.	Heparin	43.0	43.0	43.5	41.0	0.0	+2.5
Average			46.6	46.1	47.1	45.0	+0.5	+2.1

*A standard value of 0.5 volumes per 100 volumes of blood was added to the red blood cell volume.

†This case has been omitted from all averages for two reasons. First, it is a case of microcytic anemia, in which (as was stated in the original paper) the values obtained by the cell opacity method are too low. Second, because the total cells are only ⅔ of the average for total cells, the difference between the hematocrit and the dye method should be only ⅔ of the average difference between the two methods. These variations are shown quantitatively in this case.

In column 1 are given the values obtained by the hematocrit method for the volume of the red blood cells only. Hematocrit tubes were capped and spun at 3,200 r.p.m. for thirty minutes, usually in duplicate. The speed of centrifugation was checked frequently. The volumes were read ±0.2 to 0.3 per cent of the whole blood. The readings were in agreement by the independent observations of the two observers.

In column 2 are given the cell volumes as measured by the cell opacity method. These values were determined from the line given in Fig. 2, which was established as follows: The average of the volumes of red blood cells only, obtained by the hematocrit method, was plotted against the arithmetical average of the galvanometer readings for this series of determinations. The galvanom-

eter reading for plasma alone (0 cell volume) is 98. The two points were connected by a straight line.

The results of the hematocrit method and the cell opacity method are compared in column 5. With the exception of the first case the volumes by the cell opacity method correlate with those of the hematocrit method well within the limits of the agreement originally claimed for the cell opacity method.

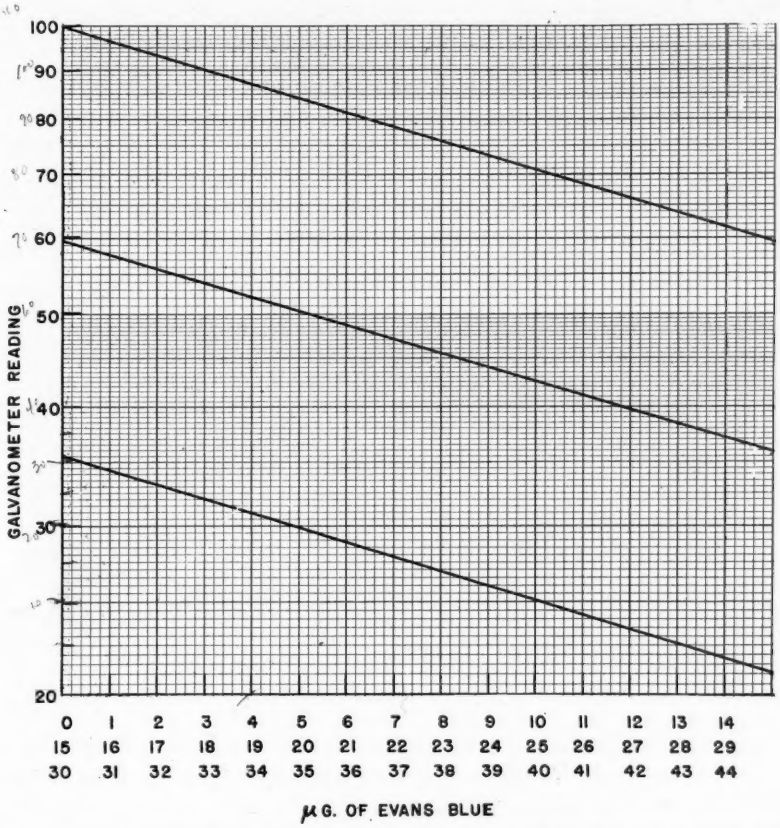

Fig. 1.—Concentration of Evans blue in plasma. This curve was made from known concentration of the dye in plasma. For each determination 0.1 c.c. of plasma was diluted to 10 c.c. with 0.85 per cent sodium chloride solution.

In column 3 are given the total cell volumes obtained by the hematocrit method. A constant value of 0.5 volumes per 100 volumes of blood was added to the red blood cell volume, as given in column 1 to obtain the total cell volume. We considered this value more accurate than actual reading of the volume of the buffy coat. We selected 0.5 rather than the more widely accepted 0.6, so that the error, if any, should make the total too small rather than too large.

In column 4 are given the cell volumes obtained by the colorimetric Evans blue method, as described under Procedure. The values were calculated from the line given in Fig. 1.

The values given in columns 3 and 4 are compared in column 6. The volumes obtained by the dye method never exceeded or even equaled those obtained by the hematocrit method, and averaged 2.1 volumes (4.5 per cent) less than the hematocrit values. Four aliquots of the same blood analyzed by the

Evans blue method have always yielded values within 1 per cent of one another (that is, 0.5 volumes of cells per 100 volumes of blood). Inasmuch as duplicate hematocrit determinations always agreed within our error of reading, and inasmuch as the absolute accuracy and causes for variation in the hematocrit method are unknown, it is impossible for us to state the difference between the two methods in accurate statistical terms. Approximate analysis shows that this value is more than five times the probable error of the difference ($\sqrt{\text{sum}}$ of the squares of the probable errors) and therefore the difference is statistically highly significant. Even if the buffy coat were not taken into account the difference would still be significant.

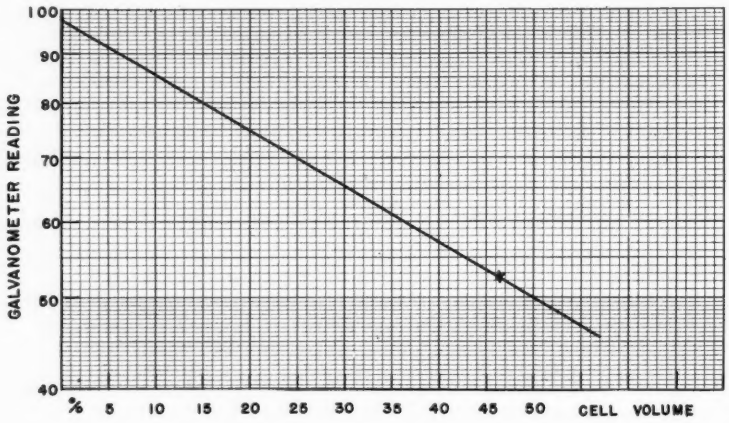

Fig. 2.—Cell volume by the cell opacity method. X = the average of the determinations in this series, which were made with 20 c.mm. of blood suspended in 10 c.c. of citrate solution and read in an Evelyn photoelectric colorimeter.

Gregersen and Schiro[8] found a difference of 4.2 per cent between the recovery of the dye added to whole blood and that recovered in the supernatant plasma following centrifugation. This value is in excellent agreement with the difference we found between the values for cell volume obtained by the dye method and by the hematocrit method, namely, 4.5 per cent.

It seems reasonable that the lower volumes obtained by the colorimetric method represent a truer value than that obtained by the hematocrit method. The red blood cells, whether they are biconcave disks, or cup-shaped, or spheroid, could hardly be packed so tightly that there would be no plasma between them. This deduction is subject to experimental verification by seeking for dye in the packed cells, which would prove the presence of plasma between them.

We added 5 c.c. of oxalated whole blood to a tube containing 4 mg. of Evans blue. This was thoroughly agitated until the dye was completely dissolved, and 4 c.c. of the blood were transferred to a tube with a constriction slightly below the 2 c.c. level. This tube and a hematocrit tube were centrifuged at 3,200 r.p.m. for thirty minutes. The packed cells extended above the constriction. The tube was scratched with a file at this point and broken off. The sticky packed cells were then transferred by repeated washings into 15 c.c. of physiologic saline solution. This was centrifuged and 10 c.c. of the clear supernatant solution, tinged blue, were removed and measured for concentration of Evans

blue. The volume of cells removed was determined by measuring the capacity of the tube which had contained them. From the volume of cells used, the concentration of dye, and the known cell volume, it was calculated that about 2 per cent of the dye was distributed within the space occupied by the packed cells. A similar procedure was used by Gregersen and Schiro[8] when they washed the cell residue with isotonic saline or dye-free plasma and recovered 3.9 per cent of the dye.

Under physiologic conditions leucocytes occupy such a small percentage of the total volume of cells that they may be neglected for most clinical purposes. By the colorimetric method the total plasma is determined, and hence the total cells. Whether the packed leucocytes contained plasma in the same proportion as the packed erythrocytes may under some conditions be an important problem. If pure white blood cells could be obtained readily, the problem could be attacked in the manner just described for red blood cells. This we have been unable to do, but we made orientation experiments. Forty to 45 c.c. of blood were centrifuged in two tubes specially constructed so that the white blood cells and platelets were contained in a constricted column. The buffy coat, admixed with less than one-half its volume of red blood cells, was removed and suspended in its own plasma. Aliquots of this mixture were taken for measurement of cell volume by the hematocrit, the colorimetric, and the cell opacity methods. The values obtained by the hematocrit method were 3.0 and 3.3 per cent for the erythrocytes and buffy coat, respectively, a total cell volume of 6.3 per cent. By the Evans blue method the cell volume was 5.0 to 5.5 per cent, and by the cell opacity method it was 3.5 per cent. As in the other experiments reported the cell volume determined by the dye method was less than that given by the hematocrit method. The determination is not sufficiently accurate to state whether more or less dye is included in the packed white cells and platelets than in the packed red blood cells. It is beyond doubt, however, that the white blood cells occupy volume as recorded by the blue dye method. This experiment also confirms the previous observations made upon the blood of patients with leucemia, that the cell opacity method gives values little influenced by the white blood cells.

DISCUSSION

The behavior of Evans blue dye in blood has been exhaustively studied by Gregersen, Gibson, and their collaborators,[8-11] who have used it successfully in the determination of plasma volume in vivo. These investigators were so interested in the use of the dye for this purpose that they did not point out the applicability of their findings to the determination of the relative volumes of plasma and cells in whole blood.

We have made the assumption that Evans blue is contained wholly in the plasma and does not enter the cells. Gregersen and Schiro[8] concluded that the dye is dissolved only in the plasma, combined with the serum protein, and that the red blood cells do not permit either penetration or absorption of the dye. In agreement with their observations we found, as detailed above, that dye contained in packed cells is readily transferred to saline solution. Further, although we used 1, 2, and 3 c.c. aliquots of blood with the same or different

amounts of dye, we have obtained the same cell volume calculated from all aliquots, within the experimental error. If the dye had been adsorbed on, or had penetrated into, the cells this finding would mean that, regardless of concentration, the same proportion of dye was held by the red blood cells, which is highly unlikely.

In determining cell volume by the hematocrit method the effort was made to have the speed of centrifugation greater than rather than less than the usual number of revolutions per minute, and thus to err on the conservative side. The larger volumes obtained by the hematocrit method cannot, therefore, be attributed to spinning the blood at too slow a speed.

The reason for making a new curve for the cell opacity method based on the present data instead of using the one previously published, was that the latter would be interpreted in terms of red blood cell volume as one or two volumes too great. The measurement of cell opacity is an independent physical measurement, and its relation to the hematocrit method is purely empirical. The absolute value for cell volume calculated from it, therefore, is theoretically not of prime importance; the main virtue of the method lies in the fact that the correlation between it and another method is close under a given set of conditions. The original data consisted of observations on a large number of children, some of which were above and some below the line adopted. In this case we have used few individuals, all adults, mostly with high cell volumes, and it may be that we have accidentally selected individuals whose cell opacity readings fell below the original line. All the methods in this study were carried out with the greatest precision. In the former study only clinical exactness was attempted. Therefore, especially for high cell volumes, this curve is more accurate. The difference between this curve and the former one decreases as the cell volume becomes smaller.

In this series of observations we have been unable to detect any consistent difference between values whether 0.2 per cent of oxalate or 1 mg. of heparin per cubic centimeter of blood was used as anticoagulant. Neither caused swelling nor shrinking of the cells sufficient to be demonstrated, even when aliquots of the same blood were compared.

Hemolysis was not visible in any samples of blood studied. If any was present, it was probably too small to affect the cell volume significantly. In any event it was the same in the aliquots compared by the three methods. In their studies of blood volume in vivo by the use of Evans blue, Gibson and Evelyn[11] found it necessary to analyze successive samples of blood in which the degree of hemolysis varied. They have shown how to correct adequately for this complicating factor. Such correction does not apply to the present study, for the dye-free plasma is used for the blank, and any interference due to hemolysis is thus eliminated. In Gibson and Evelyn's studies it was necessary to use undiluted plasma in order to obtain a concentration of dye sufficient to read in the colorimeter. Under our experimental conditions any amount of dye up to the saturation point may be used, thus eliminating the necessity for microtechnique. We used sufficient dye so that the color could be read to maximum

advantage when the plasma was diluted to 100 times its volume. This also reduced to a minimum any difficulties which might be due to any interfering substances in the plasma.

We are at a loss to account for the fact that Ponder's findings are not in agreement with ours concerning the relationship between colorimetric and hematocrit methods. He presented a table[3] which he stated "is representative of the results of many experiments." In this he compared the cell volumes given by his colorimetric hemoglobin method with those obtained by centrifuging the blood at various speeds and for various times. Two samples of blood analyzed by both methods gave values for the volume of a single cell of 85 and 78 μ^3 by the hematocrit method (spun at 4,000 r.p.m.), and 83 and 82 μ^3 by the colorimetric method, respectively. Thus each method gave values both higher and lower than the other. He did not include the buffy coat.* In our experience the colorimetric hemoglobin method yielded results more variable than did the Evans blue method. However, the hemoglobin method, as well as the Evans blue method, gave a lower average for cell volume than the hematocrit method.

SUMMARY

A colorimetric method is presented for the determination of the relative volume of the plasma and cells in blood by the use of the Evans blue dye. The error is less than 1 per cent.

By this method the total cell volume averages approximately 4.5 per cent (2.1 volumes per 100 volumes of blood) less than that obtained by the hematocrit method. This discrepancy is probably accounted for by the plasma which lies between the packed cells.

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PLASMA ALBUMIN, GLOBULIN, AND FIBRINOGEN IN HEALTHY INDIVIDUALS FROM BIRTH TO ADULT LIFE*

I. A System of Microanalysis

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FOR the past eight years we have been gathering data on the levels of the plasma proteins in healthy infants and children. In this work it is frequently necessary to use very small samples of plasma. To accomplish our task we have adapted existing analytical methods to microprocedures and have added certain techniques, all of which taken together have developed into a system of analysis for the plasma proteins that has become routine in our laboratory.

In view of the relative dependability of salting-out methods, we have based our separation of the plasma protein fractions on procedures similar to those of the "Howe technique."[1] With respect to the separation with sodium sulfate, we have found that this procedure may be carried out at room temperature and that, when filtration is used for the removal of the precipitated globulin, there is a loss of albumin seemingly due to adsorption by the filter paper. During the course of the present work, both of these findings have been reported by Robinson, Price, and Hogden,[2, 3] and the latter one has been confirmed by Harris.[4] In addition, we have found it possible to determine serum globulin, or plasma globulin plus fibrinogen, directly in samples which do not show a marked lipemia. The nitrogen content of the fractions is determined by means of a micro-Kjeldahl procedure. We have devised a distillation apparatus which may be easily and inexpensively constructed from ordinary laboratory glassware, and which has proved very satisfactory in the hands of many workers in this laboratory.

In this, as in any microanalytic work, the factor most essential to success is strict attention to detail. We are, therefore, presenting our method at some length, together with a critical analysis of the results obtained. In the near future we shall present the values obtained in our study of healthy individuals from birth to adulthood.

METHOD

A complete analysis consists of duplicate determinations of each of the following fractions: (1) total nitrogen, (2) nonprotein nitrogen, (3) fibrinogen, (4) albumin plus nonprotein nitrogen, and (5) globulin plus fibrinogen. For this, eight 0.1 c.c. samples of plasma are needed. In measuring the samples, 0.1 c.c. pipettes, which have been recalibrated, are used. The calibration is made

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on the volume each pipette will contain. Throughout the procedure, all tubes are kept tightly stoppered to avoid errors due to evaporation.

Reagents:

10% sodium tungstate, nitrogen free
2/3 N sulfuric acid, nitrogen free
Sodium hydroxide-potassium dihydrogen phosphate buffer for fibrinogen and globulin precipitants:
10.05 Gm. of potassium dihydrogen phosphate
508.0 c.c. of 0.1 N sodium hydroxide
Water to make 1 liter

According to Howe,[5] this amount of potassium dihydrogen phosphate per liter would be equivalent to 6.98 Gm. of sodium sulfate for the precipitation of fibrinogen, or 7.76 Gm. of sodium sulfate for the precipitation of globulin.

Fibrinogen Precipitant:

104.6 Gm. of sodium sulfate (anhydrous)
Buffer to make 1 liter

This solution has an effective precipitating strength of 104.6 Gm. plus 6.98 Gm., or 111.58 Gm. of sodium sulfate per liter. After dilution with plasma (0.1 c.c. of plasma to 1.9 c.c. of precipitant), the strength is 106.0 Gm. per liter.

Globulin Precipitant:

218.5 Gm. of sodium sulfate (anhydrous)
Buffer to make 1 liter

The final effective strength of the globulin precipitant after dilution with plasma and correction for the buffer salts is 215.0 Gm. per liter.

Both the fibrinogen and globulin precipitants may be slightly hazy at first, and after a few days may develop a flocculent precipitate. The solutions with the precipitates may be used, provided that 5 c.c. samples are found to be free of nitrogen after digestion, distillation, and nesslerization. Because of the buffer present, the solutions cannot be directly nesslerized.

Digestion Mixture:

Concentrated sulfuric acid (C.P.)	3 volumes
"Syrupy" phosphoric acid	1 volume

Hydrogen Peroxide, 30 per cent solution. Should not contain more than 0.025 mg. nitrogen per cubic centimeter.

Saturated Potassium Hydroxide.
Indicator:

1.250 Gm. of methyl red
0.825 Gm. of methylene blue
90 per cent ethyl alcohol to make 1 liter

The acidity of the indicator must be determined by titration with N/10 sodium hydroxide, so that the proper correction may be made in the final calculation.

Standard Sodium Hydroxide. A carbonate-free 0.1 N solution of sodium hydroxide is prepared and standardized against purified potassium acid phthalate in a concentration of 2 Gm. per 100 c.c.

Standard Sulfuric Acid. This solution should be free of ammonia, and for convenience, the normality should not be greater than that of the sodium hydroxide, since 1.0 c.c. of the solution is to be titrated with sodium hydroxide from a microburette of 1.0 c.c. capacity.

Nessler's Solution.

Stock Ammonium Sulfate, 1.0 mg. nitrogen per 1 c.c. in 5 per cent sulfuric acid.

Dilute Ammonium Sulfate, 0.04 mg. nitrogen per 1 c.c.

The solution is prepared by diluting 1 c.c. of the stock solution to 25 c.c. with water and is checked by Kjeldahl analysis of 10 c.c. portions.

*Heparin.** For plasma analysis we have used heparin as the anticoagulant.

PREPARATION OF THE SAMPLES

Total Nitrogen. One-tenth cubic centimeter of plasma is added to 1.0 c.c. of water in a 25 by 200 mm. pyrex test tube. The pipette is washed several times with the mixture and allowed to drain thoroughly. The final drop is blown out. One cubic centimeter of digestion mixture is added.

Nonprotein Nitrogen. One-tenth cubic centimeter of plasma is added to exactly 1.0 c.c. of water in a 15 c.c. pointed centrifuge tube. The pipette is washed with the mixture, drained, and the last drop is blown out. One-tenth cubic centimeter each of 10 per cent sodium tungstate and ⅔ N sulfuric acid are added. The mixture is stirred with a small glass rod, care being taken not to spread the precipitate over the tube above the fluid level. This is centrifuged (fifteen minutes at about 1,800 r.p.m.), and the clear fluid is transferred by means of a capillary tube to a clean dry test tube. Great care must be taken to avoid including any particles of the precipitate during this transfer. An aliquot of 0.7 c.c. of this is placed in a 25 by 200 mm. pyrex test tube, and 1.0 c.c. of digestion mixture is added.

Fibrinogen. One-tenth cubic centimeter of plasma is added to exactly 1.90 c.c. of fibrinogen precipitant in a 15 c.c. tapered centrifuge tube, the pipette is washed three times with the solution and drained. In adding the plasma, the tip of the pipette is placed about ¼ inch under the surface of the precipitant and allowed to drain. The plasma rises from the tip to float on the surface, and the pipette is carefully washed three times with the clear underlying fluid. In the final washing, the pipette, filled with clear fluid, is withdrawn so that the tip rests against the tube just above the fluid. The pipette is allowed to drain while being rotated against the tube, thus washing back any particles adherent to its outer surface. The final drop is blown from the end of the pipette, and the contents of the tube are mixed by rotation and allowed to stand. After standing for three hours, the precipitated fibrinogen is centrifuged down to a compact, cohesive mass. This requires ten to fifteen minutes at about 1,800 r.p.m. The clear supernatant fluid is poured off, and the tube is inverted over filter paper to

*Heparin, obtained from the Connaught Laboratories, Toronto, containing 110 units per milligram has been found most satisfactory.

drain. One and nine-tenths to 2.0 c.c. of fibrinogen precipitant are added to the tube, and the contents are mixed, centrifuged, decanted, and drained. The fibrinogen pellet is transferred quantitatively to a 25 by 200 mm. pyrex test tube by means of 2 to 5 c.c. of water. One cubic centimeter of digestion mixture is added.

In precipitating fibrinogen it was found that, although the protein had flocculated within three hours, longer standing tended to yield slightly higher values. A series of fibrinogen determinations were done on 83 samples of plasma in which two fractions of each sample were allowed to stand three hours, after which the supernatant fluids were removed and allowed to stand for twenty-one hours longer. Another set of duplicates was allowed to stand for twenty-four hours before being centrifuged. The mean value for those standing for three hours was 0.188 per cent fibrinogen, and for twenty-four hours, 0.204 per cent. In 8 of the plasma samples the increase after three hours was greater than 0.04 per cent, an amount easily detected; yet in no case was there any further salting-out of protein in the supernatant fluids removed after three hours, as would be expected if the increment were due to continued precipitation of the fibrinogen. Consequently, it was deemed possible that the increase with longer standing may be due to adsorption of other proteins upon the already precipitated fibrinogen. In a series of analyses of 5 samples of plasma, the fibrinogen was determined after intervals of one hour, two, three, six, nine, twelve, and twenty-four hours' standing. In each case there was a sharp rise to the three-hour sample, after which the curve leveled. It was, therefore, decided to standardize the procedure, using this interval of time for the fibrinogen precipitation.

Albumin Plus Nonprotein Nitrogen. One-tenth cubic centimeter of plasma is added to exactly 1.90 c.c. of globulin precipitant in a 15 c.c. tapered pyrex centrifuge tube. The addition is made in the same manner as that used for fibrinogen, and the contents of the tube are mixed and allowed to stand for three hours at room temperature. We have found that longer standing, up to twenty-four hours, does not alter the amount of globulin precipitated. The tubes are then centrifuged for forty-five to sixty minutes at 2,600 r.p.m. In all cases studied, except those showing a marked lipemia, this centrifugation has been sufficient to remove the globulin as a small, fairly compact mass, leaving a crystal-clear supernatant albumin solution. This clear solution is transferred by means of a capillary tube with bulb attachment to a clean, dry test tube. One cubic centimeter of the albumin solution is pipetted into a 25 by 200 mm. pyrex test tube, and to it 1 c.c. of digestion mixture is added. The precipitate is saved for globulin analysis.

In those cases in which the plasma shows an extreme lipemia (in our work, infants, bled one to two hours after feeding), it is difficult or impossible to remove the globulin by centrifugation. In a few such instances, the precipitate rose to the top on prolonged centrifugation, and the clear subnatant solution was removed for albumin determination. More often, however, the precipitate failed to separate completely, and it was necessary to remove it by filtration. A small (1 to 1.5 cm.) funnel was fitted with a 2.5 cm. filter paper (nitrogen free),*

*The filter paper must be tested for soluble nitrogen.

by moistening with 95 per cent ethyl alcohol and drying in a current of air. The funnel was then placed in a test tube supported inside a large, straight-walled desiccator containing globulin precipitant to a depth of one inch. The desiccator should be covered during filtration, allowing the air to maintain water equilibrium with the globulin precipitant and thus prevent evaporation from the protein solution. The solution should be repeatedly filtered through the same paper until the filtrate is clear. One cubic centimeter of filtrate is used for albumin analysis.

In cases where the solution is filtered through paper, a correction is necessary because of the albumin adsorbed on the paper. This correction is determined by running a series of albumin analyses in which the values obtained by filtration and centrifugation are compared. In a series of 50 such determinations in our work, the filtrate albumin was lower than that in the centrifuged supernatant solution by 4 per cent of the amount present in the latter sample. This figure would probably vary with the size and quality of filter paper used.* Robinson, Price, and Hogden[3] have obviated the need for this correction by first saturating the paper with albumin, then filtering a second portion of the solution through the same paper. This method was not practical in our work, since only a small amount of plasma was available for complete analysis.

Centrifugation and filtration are carried out at room temperature since, in agreement with the work of Robinson, Price, and Hogden,[2] we have found no difference between these results and those obtained when the precipitation and removal of globulin are done at 37° C. Our work was done at 25° to 35° C. Although no determinations were completed at temperatures below this point, it seems likely that the results at 20° C. would be comparable, since sodium sulfate does not crystallize from the solution at this temperature.

Globulin Plus Fibrogen. The precipitate remaining after removal of the albumin solution (by centrifugation) is washed with 2.0 c.c. of globulin precipitant. For this washing, one drop of the fluid is added, and the precipitate is first broken up and suspended in this small volume by gently tapping the tube, then the remainder of the 2.0 c.c. is added. To the mixture 0.1 c.c. of water is added. This amount of water dilutes the precipitant to approximately the same concentration of sodium sulfate which was used originally; however, its chief function is to lower slightly the specific gravity of the medium and to insure removal of the precipitate on subsequent centrifugation. With clear or slightly lipemic plasma samples the 0.1 c.c. of water is unnecessary, since the precipitate may be removed by centrifugation and the error caused by the higher concentration of sodium sulfate is negligible. With a moderate lipemia the globulin remains suspended upon centrifugation unless this water has been added to the washing fluid. The above mixture is centrifuged for forty-five minutes at 2,600 r.p.m., and the fluid is removed and discarded. The precipitate is washed a second time, centrifuged, and the washing fluid is discarded.

The precipitate of globulin and fibrinogen is now transferred to a 25 by 200 mm. pyrex test tube. Approximately 1 c.c. of water is added to the precipitate

*We have used E. and D. No. 613, smooth finish filter paper.

and allowed to stand for ten minutes or longer. This loosens the pellet so that it can be transferred as a whole to the digestion tube. The centrifuge tube is washed with three or more successive small portions of water. One cubic centimeter of digestion mixture is added to the precipitate plus washings, and the tube is heated in a boiling water bath for thirty minutes, or until it begins to show discoloration. This preliminary heating avoids excessive foaming in the subsequent digestion.

In cases where the globulin is originally removed by filtration, it is discarded and the amount present is determined by difference.

DIGESTION OF SAMPLES

Each of the tubes prepared as outlined above is treated in the following manner: Add 2 or 3 glass beads. Heat with a microburner until dense fumes of sulfur trioxide are given off. Cool. Add 2 drops of 30 per cent hydrogen peroxide, and heat as before. Cool. Add 1 drop of the hydrogen peroxide and again heat until fumes are given off. Cool, and repeat, again using 1 drop of hydrogen peroxide. Cool well and add about 10 c.c. of water.

DISTILLATION OF NITROGEN

Ammonia from each of the samples of albumin, globulin plus fibrinogen, and total nitrogen is distilled into exactly 1.0 c.c. of standardized N/10 sulfuric acid contained in a test tube of 20 to 30 c.c. capacity. The nonprotein and fibrinogen nitrogens are distilled into 1.0 c.c. of the acid in a Peebles-Lewis colorimeter tube.[6] If the Peebles-Lewis colorimeter is not used, the ammonia can be distilled into a graduated test tube and an appropriate aliquot taken for nesslerization.

Fig. 1 is a diagram of the completely assembled distillation apparatus, which is similar to that of Pregl as modified by Goebel.[7] The only important new feature is the reservoir (*I*) of redistilled water attached to tube *B* through a slightly constricted opening, as indicated in Fig. 1. We also use a current of air washed with 10 per cent sodium hydroxide and 10 per cent sulfuric acid rather than the steam distillation employed by Goebel. The inverted flask, *III*, serves as a trap and equalizes the air current through the apparatus. The entire apparatus may be easily assembled from ordinary laboratory glassware, and is mounted upon a single ring stand. Especial care should be taken to insure a close fit of the stopper *a* to the digestion tubes (*IV*) and to the tubes *A* and *B*. Any space which may fill with condensed steam serves potentially as a trap to retain ammonia. We have used a quality of stopper designated as "sulfur-free, translucent, floating stock," and have replaced the stopper at the first sign of spreading around tubes *A* and *B*, or of hardening of the rubber. Tube *B* has a 2 to 3 mm. hole in its upper surface about 2 cm. from the lower end, and just below stopper *a*. This allows a free passage of air and steam through the apparatus at the same time that condensed steam is draining back from *B* to the test tube *IV*.

The distillation procedure is as follows: Clamp the digestion tube (*IV*) in position. Place the receiver (*V*) containing 1.0 c.c. of acid in the beaker *VI* and put it into position as shown in Fig. 1. The beaker is half filled with cold water.

Fill the burette *II* by means of clamp *1*. Start the current of air with clamp *4* open; then by slowly closing *4*, the air is directed as a moderately rapid stream through the apparatus. With the air flowing, slowly add 4.0 c.c. of the saturated potassium hydroxide to tube *IV* by opening clamp *2*. After about one minute begin heating tube *IV* with a microburner, and continue heating and aeration until steam begins to enter the receiver fluid as determined by the crackling

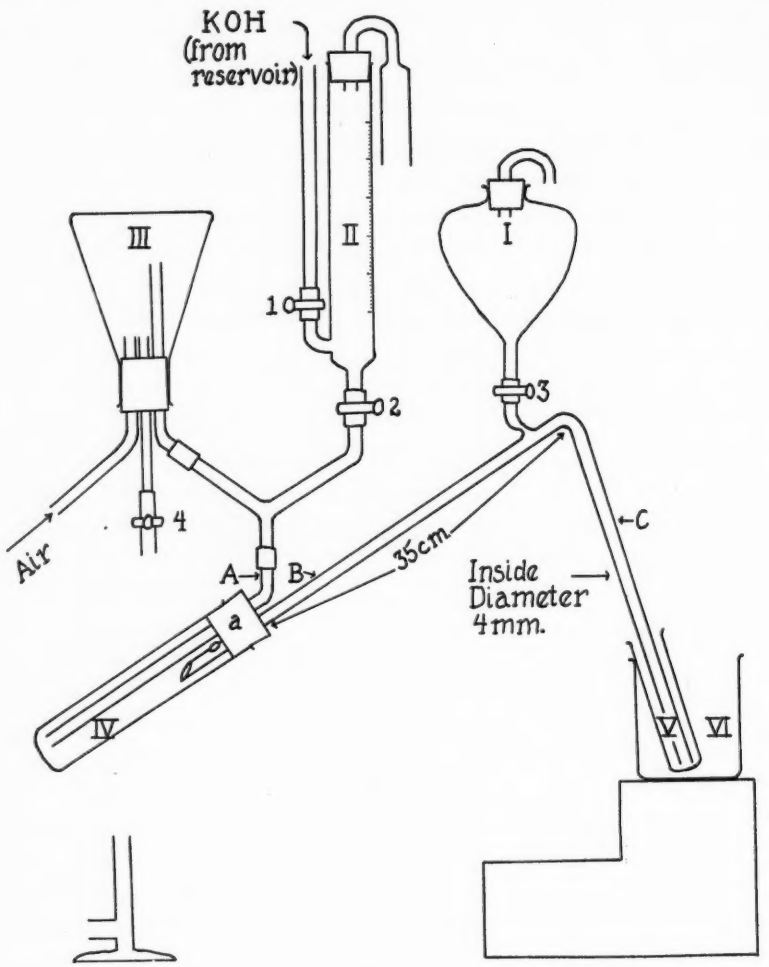

Fig. 1.

noise produced (about two minutes). At this point, remove the flame and open clamp *4*, stopping the flow of air. Lower the receiver until tube *C* is above the fluid level. If necessary, blow out tube *C* by momentarily placing a finger over the rubber tube below clamp *4*, but do not run a large amount of air through the apparatus. Wash back the ascending arm (tube *B*) of the distillation apparatus with water from *I* by opening clamp *3*. Cooling within the apparatus will suck the water back into *IV*. Now raise the receiver, start the air, and heat until steam again reaches the acid. Stop the distillation, and wash as before. Repeat this procedure once more. After the final washing of tube *B*, with the

receiver still in its lowered position, start the air and admit a small amount of water at clamp *3*. This will be blown through *C* into the receiver. Again heat tube *IV*, and continue aeration and boiling for about two minutes. The total volume of the distillate and washings should be about 8 c.c.

DETERMINATION OF AMMONIA IN THE DISTILLATES

Total-, Albumin-, and Globulin-Plus Fibrinogen-Nitrogen. To the distillate add 3 drops of indicator and titrate with standard sodium hydroxide until no further change of color is produced by the addition of more alkali. We use a modified Rehberg[8] burette of 1.0 c.c. capacity, calibrated to 0.01 c.c. The reading is estimated to 0.001 c.c.

Nonprotein Nitrogen and Fibrinogen. The distillate in the Peebles-Lewis colorimeter tube is made up to 8.0 c.c. with water. To this are added 2.0 c.c. of Nessler's solution, and comparison is made with standards prepared at the same time as the unknown solution. Two standards are prepared as follows:

	I	II
Ammonium sulfate (0.04 mg. nitrogen per cubic centimeter)	1.0 c.c.	2.0 c.c.
Water to make	16.0 c.c.	16.0 c.c.
Nessler's solution	4.0 c.c.	4.0 c.c.

In making the color comparison, the unknown is left at a volume of 10 c.c., and the standard is adjusted to match it. The volume required is recorded.

With extremely high values for nonprotein or fibrinogen nitrogen, it is necessary to use an aliquot of the distillate; therefore, it is advisable to add Nessler's solution to only one of each pair of duplicates until the approximate ammonia concentration has been determined.

DETERMINATION OF BLANKS

Two cubic centimeters of digestion mixture and 8 drops of 30 per cent hydrogen peroxide in a 25 by 200 mm. pyrex test tube are heated to dense fumes of sulfur trioxide. This is cooled well, and 10 c.c. of water are slowly added. Duplicate samples are distilled for titration and for nesslerization, using 8 c.c. of saturated potassium hydroxide. The distillate for nesslerization is made up to 8 c.c., and 2 c.c. of Nessler's solution are added. This is compared with a standard containing 1 c.c. of ammonium sulfate, 23 c.c. of water, and 6 c.c. of Nessler's solution. The value found is divided by 2 to obtain the correction blank.

We have found the ammonia blank for these reagents to equal the acid blank of 2 to 4 drops of indicator, and for convenience we have adjusted the amount of indicator between these limits to cancel the ammonia blank in the samples for titration.

CALCULATION OF RESULTS

Nonprotein Nitrogen:

$$\frac{\text{Reading of standard}}{20} \times \text{mg. nitrogen in standard} = \text{mg. nitrogen in distillate.}$$

$$\frac{(\text{Mg. nitrogen in distillate} - \text{blank}) \times 1.3 \times 100}{\text{Volume of plasma sample} \times 0.7} = \text{mg. nitrogen per 100 c.c.}$$

Fibrinogen. The calculation is similar to that for nonprotein nitrogen. The value for milligrams nitrogen per 100 c.c. is converted to milligrams protein by multiplying by the factor 6.25; this is divided by 1,000 in order to express the final result in terms of grams.

Total Protein:

c.c. N/10 sulfuric acid + indicator blank − c.c. N/10 sodium hydroxide − ammonia blank = c.c. N/10 ammonia distilled.

$$\frac{\text{c.c. N/10 ammonia} \times 1.4 \times 100}{\text{Volume of plasma sample}} = \text{mg. total nitrogen per 100 c.c.}$$

$$\frac{\text{Mg. total nitrogen} - \text{nonprotein nitrogen}}{1{,}000} \times 6.25 = \text{Gm. protein per 100 c.c.}$$

Albumin. The calculation is similar to that for total protein, except that the value for milligrams nitrogen must be multiplied by 2 since an aliquot equal to one-half of the albumin solution was used.

Globulin Plus Fibrinogen. The calculation is similar to that for total protein, except that no correction is made for nonprotein nitrogen.

REPRODUCIBILITY OF RESULTS AND A CONSIDERATION OF THE "SALTING-OUT" PROCEDURE

The accuracy of the Kjeldahl procedure may be judged from the results obtained in a series of 24 analyses of the total nitrogen in 0.1 c.c. fractions of a single sample of plasma. The 24 determinations gave a mean value of 1.187 mg. nitrogen, with a standard deviation of 0.011 mg. nitrogen. The highest value obtained, 1.220 mg., is 0.033 mg., or three standard deviations above the mean, and the lowest value, 1.168 mg., is 0.019 mg., or about two standard deviations below the mean. The results obtained on a series of 100 consecutive complete determinations were also analyzed statistically.[9] The standard deviation and standard error of the difference between duplicates for each fraction are given in Table I.

TABLE I

ACCURACY OF THE METHOD

NITROGEN FRACTION	STANDARD DEVIATION OF DIFFERENCE	STANDARD ERROR OF DIFFERENCE
	Gm. protein per 100 c.c.	Gm. protein per 100 c.c.
Total protein	0.072	0.101
Albumin	0.077	0.109
Globulin + fibrinogen	0.035	0.049
Fibrinogen	0.0087	0.0123
	Mg. nitrogen per 100 c.c.	Mg. nitrogen per 100 c.c.
Nonprotein nitrogen	1.123	1.742

In routine analyses a value obtained in a single determination should be within two standard deviations of the true value in 95 per cent of the cases. When duplicates are run, the results should differ by not more than two standard errors in 95 per cent of the cases. Thus, a single determination of the total protein by this method will give a value within ±0.15 Gm. per 100 c.c. of the true value in 95 per cent of the analyses run. Similarly, it is unlikely that the value obtained for albumin is in error by more than ±0.15 Gm.; for globulin plus fibrinogen, ± 0.07 Gm.; for fibrinogen, ± 0.017 Gm.; and for nonprotein nitrogen, ±2.5 mg. per 100 c.c.

This concept of the accuracy of the method is based upon the assumption that an exact separation of the different protein fractions has been accomplished by the salting-out procedure. It is well known that a single "salting-out" will not quantitatively separate the two types of plasma protein, the albumin and globulin. In order to avoid this difficulty and still preserve the significance of analytical work, the terms "albumin" and "globulin" have come to mean those fractions of the plasma proteins which are separated by a single precipitation with sodium sulfate at a specific concentration and under controlled conditions. In the present work we have followed this accepted usage of the terms.

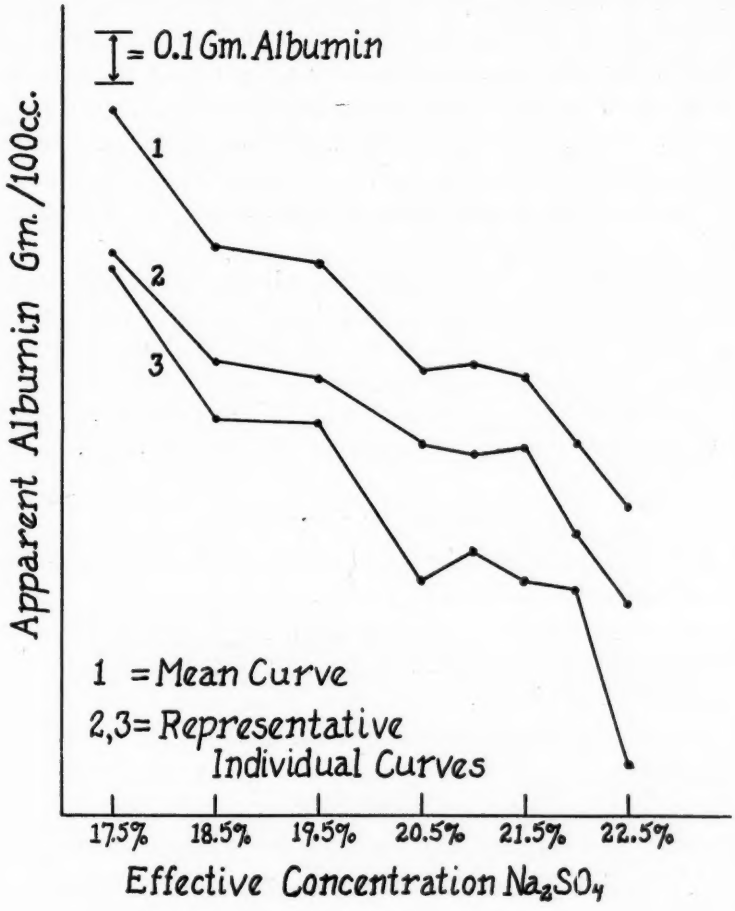

Chart 1.

Our choice of definitive conditions and concentrations of sodium sulfate is based largely upon the findings reported by Howe. This author[10] has shown that the separation of fibrinogen with 10.6 per cent sodium sulfate gives values comparable to those obtained by other methods. In our work we have found that sodium sulfate in this concentration does not remove any of the protein from serum. It can, therefore, be concluded that no fraction other than fibrinogen will be separated from plasma unless it is adsorbed by the fibrinogen. In studying the protein precipitation with increasing concentrations of sodium sulfate,

Howe[11] found a critical zone between 20 and 22 per cent with plasma dilutions of 1:10. In our work the plasma dilution has been 1:20; however, this greater dilution has not produced a significant shift in the critical zone, as can be seen in representative curves given in Chart 1. In the curves of this chart an additional critical zone is shown at 18.5 to 19.5 per cent sodium sulfate, which was not found with some plasma samples. In all cases studied, covering a wide range of absolute values, the curves in the region of 21.5 per cent sodium sulfate were similar to those shown. Since this strength of sodium sulfate is usually employed for globulin precipitation, and does fall within the critical zone, we have continued to use a 21.5 per cent solution of the salt. Judging from the curves of Chart 1, it would seem that the use of 21.0 per cent sodium sulfate may be preferred, since this concentration falls more nearly at the center of the critical zone. It is probable that analytical results would not be significantly altered by this change. Although the separation of globulin and albumin is not complete, the values obtained on a given sample of plasma can be reproduced with considerable accuracy, and those on different samples of plasma should be comparable.

Chorine[12] has shown that the apparent plasma protein concentration may be markedly influenced by the nature and the amount of the anticoagulant used. According to this author's work, as well as that of others, heparin produces at most only a very slight change in plasma concentration. We believe, therefore, that our results give a true estimation of the amount of the circulating plasma proteins, unaltered by the anticoagulant which is added after the blood is drawn.

SUMMARY AND CONCLUSIONS

1. A method is presented for the separation and Kjeldahl analysis of the fibrinogen, albumin, and globulin of plasma.

2. A complete analysis of total nitrogen, nonprotein nitrogen, fibrinogen, albumin plus nonprotein nitrogen, and globulin plus fibrinogen may be done in duplicate on 0.8 c.c. of plasma.

3. A simple but accurate modification of existing micro-Kjeldahl procedures has been devised.

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AN IMPROVED RAT CAGE FOR METABOLISM STUDIES*

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DURING the last few years the well-known wire drum and glass funnel metabolism cages have been in use in this department, but just as in other laboratories, we found that this cage had many disadvantages (contamination of feces and urine with food, washing of feces by urine, evaporation of urine, etc.). In the course of time many improvements have been made by members of this laboratory, using similar cages for metabolic studies, and gradually an entirely new type of cage was developed which eliminates most of the disadvantages mentioned. Since this metabolism cage proved very satisfactory, we wish to describe it here briefly in the hope that it will prove useful to other investigators as well.

Fig. 1 shows the complete unit of six cages with all parts assembled, as it appears when in use.

Fig. 2 shows the individual parts:

Cage. 7 by 8 by 5 inches of galvanized iron with wired top edge, having a hinged wire mesh door or lid (4 to 1 inches) fastened by means of a bronze door clip. The base of the cage is of wide wire mesh as indicated, to allow feces to drop through. Two outlets 1⅜ inches in the front end of the cage enable the rat to obtain food and water. These holes must be placed high enough in the wall of the cage so that it becomes necessary for the rat to place its front paws on the edge of the hole when it takes food. This arrangement prevents the animal from lifting food into the cage where it would become mixed with excrements. Therefore, the height of the holes must be chosen according to the size of the rat. Above, center, is a small hole into which fits a rivet on the diet box which holds the box securely fastened to the cage front. An angle rest (as shown), front and back, supports the cage on the rack.

Diet Box. This box 5½ by 2⅛ by 4 inches of galvanized iron is divided into two compartments. One forms a food bin of wire mesh for gross food; the other, with a dividing shelf with hole in center, allows tip of water bottle to pass through; this shelf acts as a support for the bottle and prevents the eating of

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the rubber stopper by the rat. Below these two compartments is sufficient space to insert a two-compartment drawer which is used to hold ground food on one side and to catch drips from the water bottle on the other. If extreme accuracy in determining of food intake is required, only ground food should be admin-

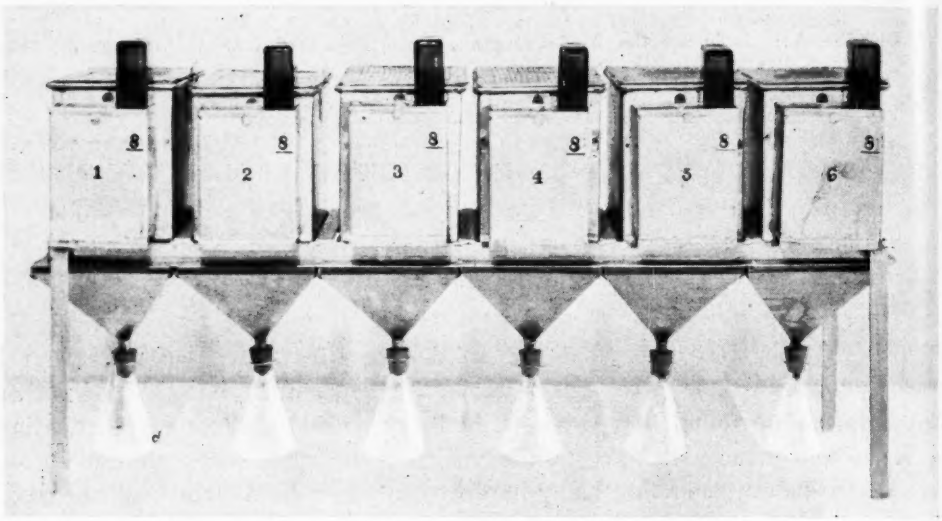

Fig. 1.

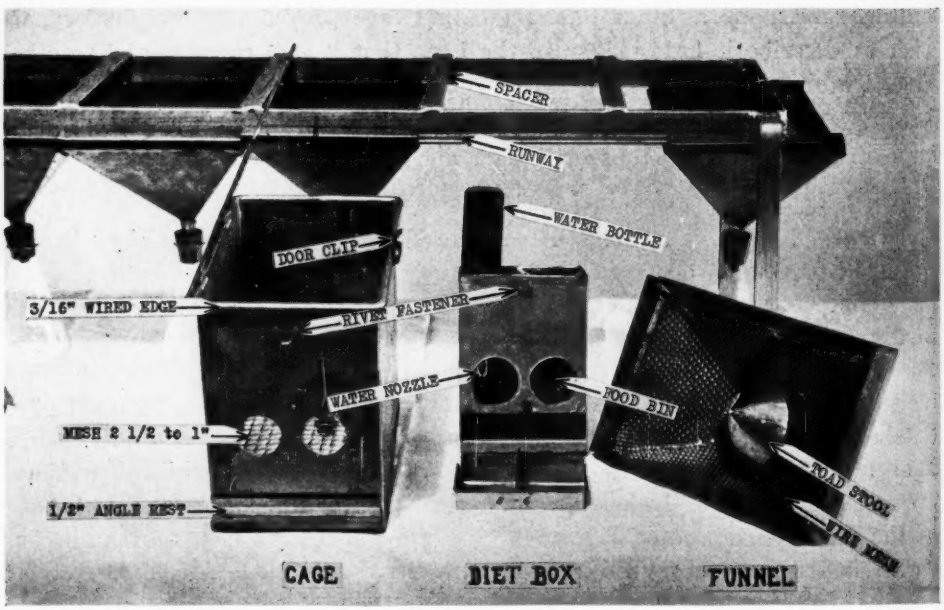

Fig. 2.

istered, since this can be measured exactly before and after the experiment by weighing the contents of the drawers. When the animal is being fasted, a small tray replaces the food drawer, fills in the space, and prevents the rat from crawling through the outlets into the diet box and the consequent loss of urine.

Rack. Legs are of ¾ by 3/16 inch flat bar welded to 1 by ½ inch angle iron; runway for funnels of 1 by ½ by ⅛ inch angle iron extending from end to end of the rack. Flat bar spacers ¾ by ⅛ inch are welded to side bars front and back, spaced along the length of the rack at a distance of the width of each cage to prevent cages from sliding out of position over the funnels.

Funnels. Constructed of monel metal throughout are pyramidal in shape. Inserted in each funnel is a monel wire mesh rack similar in shape but somewhat less deep than the funnel and having a monel "toad stool" in the center. The toad stool is so constructed that the feces dropping from above roll off and collect in the rack at the base. The urine runs off the stool and drips directly through the mesh into the deeper funnel below without washing the feces. The funnel drains into an Erlenmeyer flask fastened by means of an airtight rubber stopper placed over the end of the funnel drip tube. This arrangement keeps evaporation of urine at a minimum.

As will be seen a supporting rivet is placed on both sides of the diet box; the use of one has already been described. The second is used when both fasting and thirst are desired. In this case it is possible to turn the diet box around, thus presenting a solid front toward the rat and covering the outlets of the cage.

The main advantages of this cage are:

(1) No contamination of feces and urine by food.

(2) No washing of feces by urine.

(3) Achievement of extreme accuracy in measurement of both food and water intake.

(4) Minimum urine evaporation.

(5) The cages are relatively inexpensive because they are unbreakable and rust proof.

MEDICAL ILLUSTRATION

THE PHOTOGRAPHIC ASPECT OF LIGHT REFLECTION FROM HUMAN SKIN*

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CONSIDERABLE study has been given to the transmission and penetration of light *through* human skin, but it would appear from the literature that little attention has been given to the photographically important question of *reflection* of light from human skin. Experience reveals wide variations in the photographic appearance of skin taken under different conditions. (1) Such variations are attributed to the color sensitivity of the emulsion used, the color temperature of the light source, and/or the use of color filters. These explanations are inadequate since they place the subject on a par with the photography of colored objects and their translation into monochrome; they do not account satisfactorily for the need to use make-up to secure good photographs in color of the human face, and still less do they explain the cause of the variations in texture and definition shown by photographs of human skin taken by reflected light of different color temperatures. (2) More particularly, they afford no understanding of why skin photographs are waxlike under red light and show crisp definition under blue light.

The thesis of this paper is that human skin is a living tissue with definite characteristics with regard to the response of different wave lengths to light, and that the true explanation of the differences shown in photographs is to be found by ascertaining the *level* from which reflection of light is taking place. The basic point of this thesis is that different wave lengths of light are reflected from different levels (depths) of human skin, and this introduces four additional factors: penetration, absorption, reflection, and scatter, consideration of which is necessary for a complete explanation of the results obtained.

Skin varies considerably in thickness in different parts of the body, e.g., cross sections from the back show greater thickness of the stratum mucosum than similar cross sections from the back of the hand (Fig. 1, layer C). Similarly, skins from the palm of the hand or sole of the foot vary in thickness, and there is thus no one area standard for the whole body. For the purpose of experiment, skin from the back of the human hand was used throughout.

Fig. 1 is a photomicrograph of a transverse section of skin from the back of a hand of a Chinese and shows its structure and various layers. On it are shown the approximate levels at which it would appear by experimental photo-

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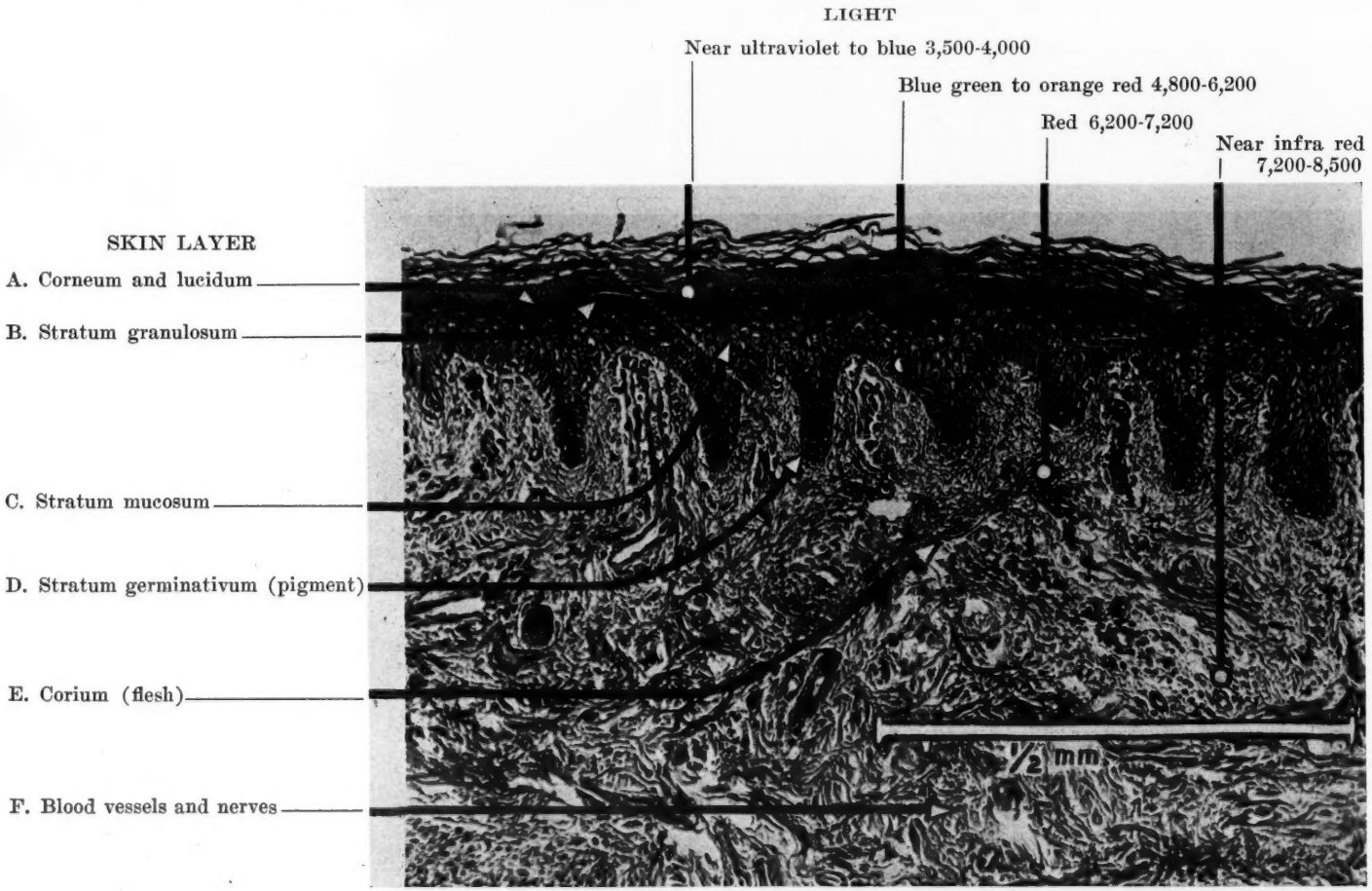


Fig. 1.—Photomicrograph of a transverse section of skin from the back of the hand of a Chinese, showing its structure and various layers. On it are seen the approximate levels at which it would appear by experimental photographs that the light used was reflected.

graphs that the light used was reflected. Such levels must necessarily be approximate only, because, as previously stated, human skin in any event varies in thickness.

Experiments in photographing through skin and flesh indicate[2] that a powerful beam of light has considerable penetrating power and that, as is logical, the stronger and more concentrated the light, the greater the penetration and transmission. Such experiments further reveal that the transmission is mostly toward the red end of the spectrum and progressively decreases as it approaches violet and ultraviolet.[3-8] This confirms results obtained photographically by reflected light.

TABLE I

FIGURE NO.	HAND	PLATE USED	ILLUMINATION USED	FILTER USED
2	European	Special rapid	Quartz mercury vapor	Tri-blue
3	European	Special rapid	Half-watt	Tri-blue
4	European	Panchromatic	Half-watt	None
5	European	Panchromatic	Half-watt	Beta
6	European	Panchromatic	Half-watt	Tri-red
7	European	Infra-red	Half-watt	Infra-red
14	Indian	Special rapid	Quartz mercury vapor	Tri-blue
15	Indian	Infra-red	Half-watt	Infra-red
8	European and Chinese	Infra-red	Half-watt	Infra-red
9	European and Chinese	Panchromatic	Half-watt	Tri-red
10	European and Chinese	Panchromatic	Half-watt	None
11	European and Chinese	Special rapid	Half-watt	Tri-blue
12	European and Chinese	Special rapid	Quartz mercury vapor	Tri-blue
16	Case of pellagra, Chinese	Special rapid	Daylight	Tri-blue
17	Case of pellagra, Chinese	Panchromatic	Daylight	None
18	Case of pellagra, Chinese	Infra-red	Daylight	Infra-red

To demonstrate the correctness of our thesis a series of photographic experiments was carried out, using the following Ilford plates and filters, with a quartz mercury vapor lamp, half-watt lamps, and daylight. The plates used were:

Ilford "special rapid," an ordinary or noncolor-sensitive plate of medium speed, sensitive from about 3,600 to 4,950 A.U., which is near ultraviolet to greenish blue. With this plate an Ilford tri-color blue filter cutting at 5,150 A.U. was used; thus, near ultraviolet and all the blue were used for taking the photograph.

Ilford "special rapid panchromatic," an orthopanchromatic type with the same development constant as the "special rapid," but sensitive from about 3,600 to 7,200 A.U. With this a beta filter was used once as a correction filter, because with half-watt light this gives practically full correction. This filter is a pale greenish yellow with a factor to H.W. of $\times 1\frac{1}{2}$ only.

Ilford "infra-red," an infra-red sensitive plate which, when used with its appropriate filter, records the action of rays between 7,000 and 9,000 A.U., with maximum sensitivity at about 8,100 A.U. The filter permits scarcely any

visible light to pass, and it is necessary to focus by a deep red filter and compensate by a slight increase in bellows extension when using it. The exact amount is 1/200 of the focal distance of the lens.

All plates were backed and thus proof against halation. A Goerz Dagor F:6.8 – 8¼ inch lens was used, stopped down to F:22. Figs. 2 to 12 were taken with a vertical camera in a darkened room without any other light than that indicated; Figs. 16 to 18 were taken in a hospital ward by daylight, the patient being in bed and too ill to be moved. Development of the special rapid and special rapid panchromatic plates was routine, a developer of the D.76 type being used, with time and temperature for contrast of about 0.9 gamma. Exposures were based on meter readings, and prints were made on Kodak "Nikko" paper of medium contrast; this corresponds to grade 2: P.M.C. paper. No attempt was made to depart from the usual procedure and no contrasts were forced up or down by interference with normal development or printing.

Table I lists the experiments, and the illustrations show the photographic differences in skin-rendering.

RESULTS

Figs. 2 to 18 show definite changes in the photographic rendering of skin texture. The longer the wave lengths employed, the greater the loss of fine detail in the skin. The results are set forth here as a record because it is impossible for a half-tone reproduction to show the gradual changes as clearly as can be seen in the originals.

FIG.	APPEARANCE
2	Minutest lines and details of skin texture are clearly recorded; more is recorded than can be seen in daylight by the eye aided by a low-power magnifying glass.
3	This shows excellent rendering of texture which is slightly inferior to Fig. 1.
4	The general appearance is excellent, but the texture is not as crisp as in Fig 1.
5	The skin is commencing to show less texture.
6	The typical waxy appearance of the skin is caused by excess red light; the texture is poor.
7	Texture has completely disappeared; subcutaneous veins are visible.
14	This excellent skin texture shows the finest details.
15	There is complete absence of surface texture of the skin; underlying veins show through.
8	No texture can be seen but the veins are clearly visible.
9	Shows waxy appearance, with little texture and detail.
10	The general appearance is excellent, but crispness of lines is lacking.
11	Crispness of lines has reappeared, especially in the skin of the Chinese.
12	Minutest lines and details of skin texture are shown. Note the higher absorption of short waves by the hand of the Asiatic compared with that of the European.

The end differences shown are great. To make direct comparison between photographs taken by blue and by red light more simple, as occurs in tri-color separation negatives of human skin, the portion of skin around the knuckles

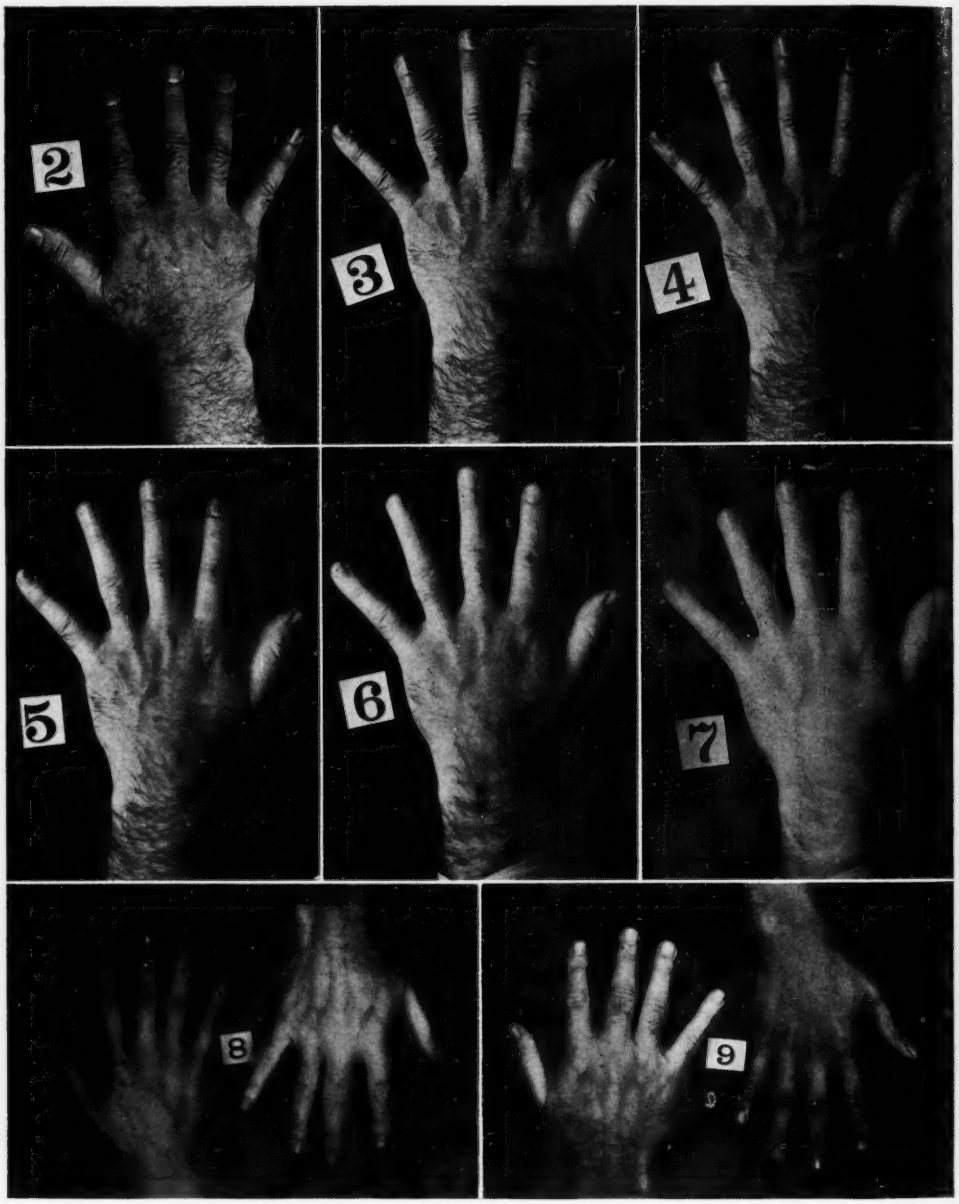


Fig. 2.—Ultraviolet on special rapid plate.
Fig. 3.—Tri-color blue on special rapid plate.
Fig. 4.—Panchromatic, half-watt, no filter.
Fig. 5.—Panchromatic, half-watt, beta filter.
Fig. 6.—Panchromatic, tri-color red filter.
Fig. 7.—Infra-red hand of European
Fig. 8.—Infra-red, European and Asiatic.
Fig. 9.—Tri-red on panchromatic, European and Asiatic.

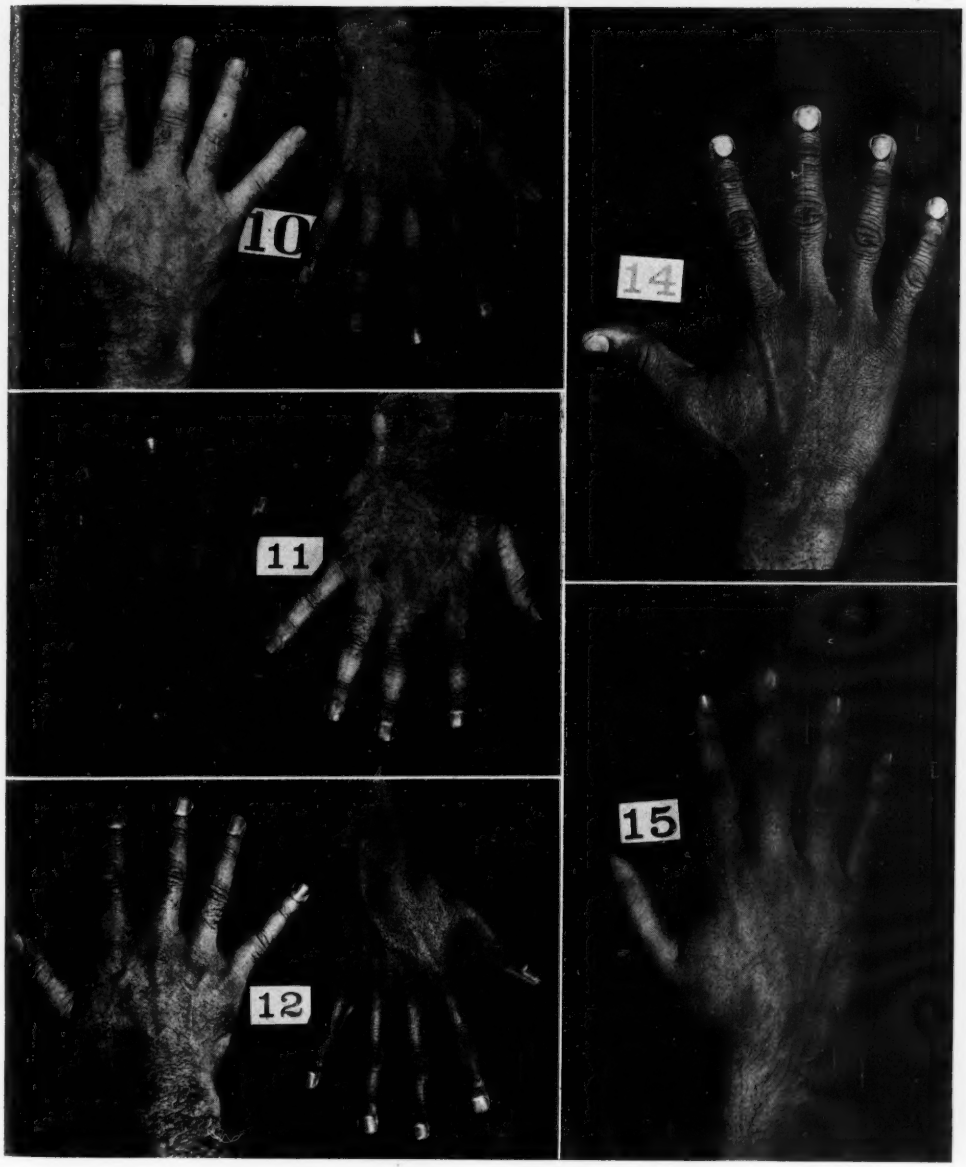
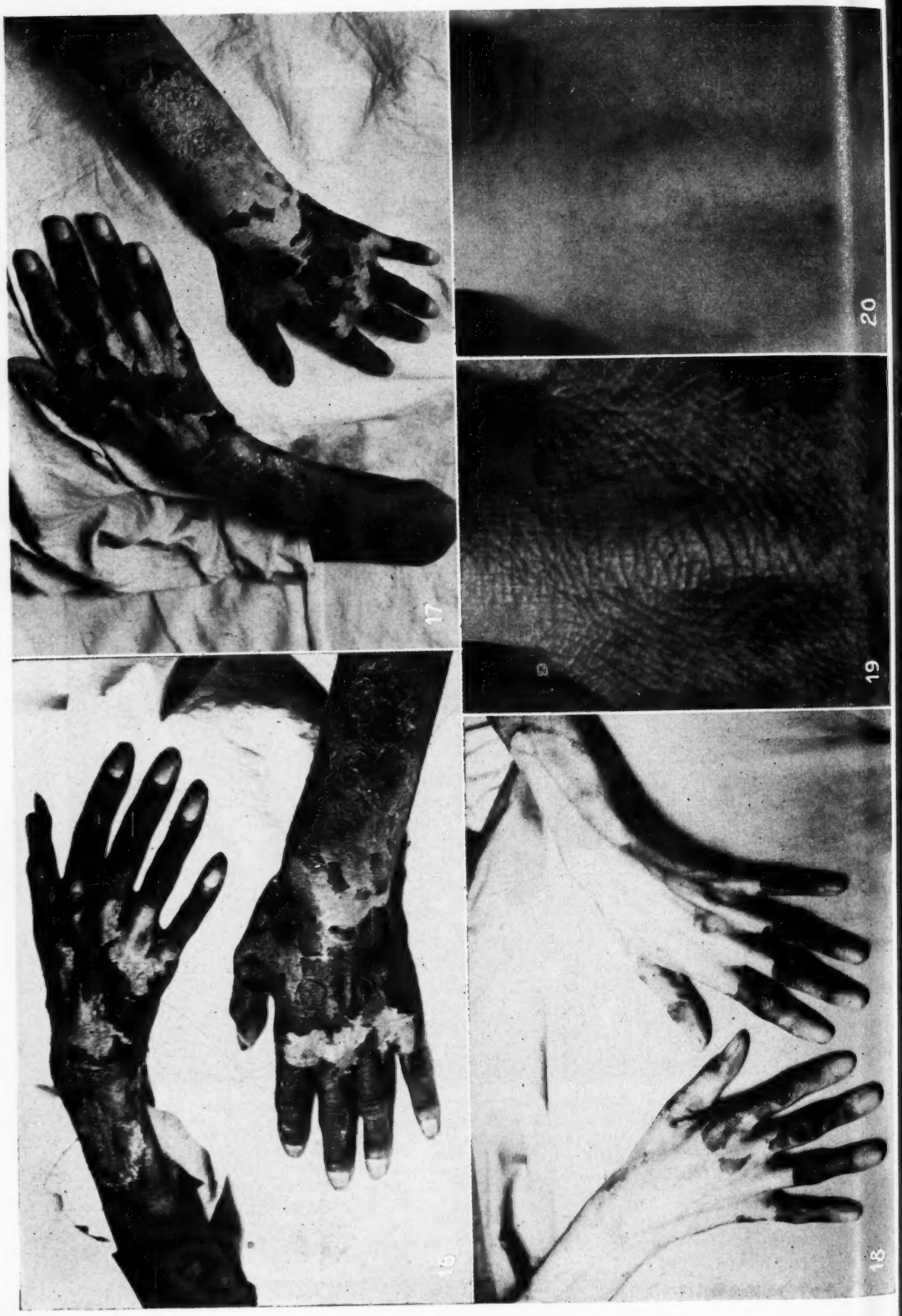


Fig. 10.—Half-watt with panchromatic, European and Asiatic.
Fig. 11.—Tri-color blue, European and Asiatic.
Fig. 12.—Ultraviolet, European and Asiatic.
Fig. 14.—Ultraviolet, Indian hand.
Fig. 15.—Infra-red, Indian hand.



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of the middle and third fingers in Figs. 3 and 6 have been enlarged and are shown in Figs. 19 and 20. These are not the extremes, which would be Figs. 2 and 7, 14 and 15, and 9 and 12. Figs. 3 and 19 show the approximate effect secured in normal sunlight by using an ortho film, whereas Figs. 6 and 20 are those secured on a hypersensitive or type 3 panchromatic film by nitrophot lights, or lights abundant in red rays.

These two comparisons are, therefore, the most important from a practical photographic aspect, since the conditions are commonly met with in ordinary practice.

DISCUSSION

The photographs reveal that as the wave length of light used for illumination is increased, the surface texture that can be photographed is progressively diminished and finally entirely lost. This can only mean that reflection of the rays that are actually making the photographic negative is taking place from different levels of the skin. Therefore, the photographic rendering of human skin is not solely a matter of color correction and color sensitivity, but is closely connected with the inherent response of skin as a living tissue to light thrown upon it.

Figs. 16, 17, and 18 are comparison photographs of the skin condition on the hands of a patient with pellagra. The panchromatic version (Fig. 17) is a good representation of the visual appearance. The blue plus near ultraviolet version (Fig. 16) accentuates surface lesions and reveals more than the eye sees; the infra-red version (Fig. 18) shows that the true extent of serious damage is limited to the fingers. It is obvious that such photographs are of great diagnostic value.

Table II sets forth an interpretation of the results of the experiments.

TABLE II

LIGHT	ABSORPTION	REFLECTION LEVEL	REFLECTION INTENSITY	SCATTER
Near ultraviolet to blue	4	A to B	1	1
Blue green to orange	3	B to C	2	2
Red	2	D	3	3
Near infra-red	1	E and F	4	4

The numbers 1 to 4 are arbitrary indicators for the amount absorbed, reflected, and scattered, as follows: (1) minute; (2) little; (3) moderate; (4) considerable. For practical purposes it is sufficient to divide the light into these four divisions, and in any case the varying thickness of skin makes exact measurements valueless.

Legends for Figs. 16-20.

Fig. 16.—A case of pellagra in a Chinese taken on a special rapid plate with daylight illumination.

Fig. 17.—A case of pellagra in a Chinese taken on a panchromatic plate with daylight illumination.

Fig. 18.—A case of pellagra in a Chinese taken on an infra-red plate with daylight illumination.

Fig. 19.—This illustration is an enlargement of the center section of Fig. 3. It was taken on a special rapid plate through a tri-blue filter with the half-watt light as illumination.

Fig. 20.—An enlargement of the center section of Fig. 6. This was taken on a panchromatic plate through a tri-red filter with the half-watt light as illumination.

It was mentioned earlier that the stronger and more concentrated a beam of light, the greater its transmission through skin and flesh. This introduces yet another factor, the strength or volume of light employed on a given area. The latter has a distinct effect on photographic rendering. This would appear to furnish one of the reasons why overlighting is disastrous to good photographs of skin texture, since evidently it results in abnormal penetration of light into the skin, with a consequent accentuated reflection from the lower layers causing excessive scatter.

Further, these experiments show clearly that the modern hypersensitive panchromatic film (type C) used in conjunction with half-watt light is not the ideal combination for work on skin. A study of the old photographs published in textbooks reveals that in general they are superior as records of skin to some more recent examples. In this sense there has been retrogression in the technical photography of skin texture.

In photographing human skin for medical records it is of paramount importance that the visual appearance of the texture and skin lesions present be clearly recorded. This is particularly necessary when a specific pathologic condition is to be illustrated, for unless the diseased condition is plainly shown the photograph is worthless.

Both normal and diseased skins have texture, but the recording of this texture is not achieved photographically unless basic requirements to secure it are known and fulfilled. Medical photography is decidedly different from portraiture; the latter aims at the suppression of defects which it is the precise function of the biologic photograph to record. The approach to the two types of subject is, therefore, entirely different.

The surface of human skin is covered with a multiplicity of fine criss-crossed lines, thus giving it the marked texture characteristic and typical of its appearance. Skin is a good reflector of normal daylight; it reflects more of the longer waves and absorbs more of the shorter, and strongly polarizes light reflected between the angles of 32 and 37 degrees. The elimination of reflections by polarizing screens destroys its living appearance and affects the recording of texture; this is evidence that it is solely the reflected light which governs the photographic appearance of skin.

The two basic factors are thus inequalities of surface and variations in reflection. Illumination must take both into consideration and so light the subject that the minute hills and dales of the skin surface are not flattened by being flooded with light. The quality of light must be such that reflection is taking place from some known portion of the skin, that is, at some known position with regard to its surface. The first requirement is standard for all photography; illumination must be so arranged that the subject is shown properly. The second is generally ignored, but is of fully as great importance. It is generally assumed that the problems are much the same as with portraiture and that, provided the lighting is directionally correct and a suitable emulsion is used, results will be as good as can be secured. This is not so.

Light thrown on human skin penetrates it, and is absorbed and reflected by it, the degree of penetration, absorption, and reflection depending upon its component wave lengths. The photographic definition secured in a negative

is considerably affected by the amount of penetration taking place and the consequent depth of reflection from the inner surfaces of the skin. This has little connection with color-rendering into monochrome as such, for the problems are specific to human skin.

Penetration plus reflection obviously causes scatter, depending upon the depth of the intervening layers of skin which have been traversed by light. The deeper this penetration, the more scattering will be the reflected rays, since such rays are scattered not only on entering but also on being reflected back to the surface; hence the difference in appearance of the same skin photographed by light of different spectral quality.[9] It is clear that an understanding of the cause of this difference enables the securing of exactly the type of photograph required.

It can be accepted as a fact that penetration through skin of the light of the complete visible spectrum, which for the purposes of this paper is taken to include the near ultraviolet and near infra-red rays, is greater as the wave length of the light increases.[3] Much work has been done along these lines, with interesting results confirmed by practical photographic experience.[4] Normally, photography is entirely dependent upon reflected light; therefore, the reflection levels rather than absorption and transmission are photographically of greater importance.

Light has a threefold action on impact with skin: absorption, penetration, and reflection. Photographically, light absorbed by a subject is lost and of no value. Since the longer wave lengths penetrate more deeply, those that are not absorbed must necessarily be reflected back to the surface from the maximum depth of penetration, and this is the light used to make the photograph and govern the sharpness of the image secured. That is, other things being equal, the photographic rendering of skin is fixed definitely by the quality of light used in the making of the photograph, and it is impossible to change this fundamental record by any subsequent chemical tinkering with the negative or print. It should now be clear that it is not a matter of color-rendering as such, because texture and definition have no direct connection with the interpretation of color into monochrome.

Although absorption is outside our subject, it is photographically important because it not only affects exposure time but also governs the color-rendering. When there is much absorption, the subject photographs dark, and vice versa. A good example of this is green vegetation photographed by blue light (or on a noncolor-sensitive emulsion) as compared to the same subject photographed by infra-red. Green leaves absorb ultraviolet and deep blue but reject deep red and infra-red; therefore, the first photograph will show the subject dark and the second will show it light in tone.[10, 11] An even more striking example is a comparison between the brown fur of a dog and the brown feathers of birds; visually there is little difference in color, but photographically there is a wide difference in exposure time required owing to the absorption of ultraviolet by brown fur and the strong reflection of ultraviolet by feathers. Measurements with photoelectric meters reveal this difference and the camera confirms it. Nature has given the animals protection against receiving insufficient or too large doses of ultraviolet. The bird is exposed to too much while flying in the

sky, whereas the animal needs all the chemical rays it can get to maintain its supply of vitamin D. There is unquestionably a similar protective system at work in the skin of man, which accounts for the selective absorption of light and its variation in the different races of mankind according to the climatic conditions under which he lives.

There are three methods of controlling the quality of light used in the making of a photograph:

1. The interposition of a light filter at some point between the light source and the sensitive film.

2. The use of light sources rich or deficient in certain wave lengths.

3. Selection of the rays by the choice of film.

The final negative quality and photographic rendering of skin will be strongly affected by these fundamental factors. It is notorious that the use of modern extreme, red-sensitive emulsions with artificial light abnormally rich in the longer wave lengths gives a waxy, textureless appearance to the skin, and our contention is that the reason for this is reflection from beneath the skin surface.

It is an interesting speculation that there may be an interference effect at the skin surface which may produce *stationary waves* somewhat along the lines of the Lippmann process of color photography.[12]

SUMMARY

The photographic appearance of human skin is modified by the reflection depths of the component wave lengths of the light used for illustration, and as such it is not a matter of color correction, as ordinarily understood, but the level of penetration and consequent reflection and scatter of the light used. Evidence is offered in the form of a series of photographs taken by various illuminants.

I wish to thank Professor Bernard E. Read, acting director of the Henry Lester Institute of Medical Research, for his help in the writing of this paper, and Dr. L. S. Kau, Tissue Pathologist of the Institute, for supplying the sections of skins required.

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DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

SULFAPYRIDINE, Level of in Blood of Children Following Dosage by Weight, Cullen, G. E., and Wilson, A. T. Am. J. Dis. Child. 60: 891, 1940.

A simple and convenient standard system of dosage of sulfapyridine on the basis of body weight is recommended: 1 grain per pound of body weight per twenty-four hours, with an initial dose of half the calculated twenty-four-hour dose and with the total daily dose not to exceed 80 grains.

Groups of infants and children of different ages have, on the average, approximately the same levels of free sulfapyridine in the blood when they are given the same dosage according to body weight.

Individual patients receiving sulfapyridine in a dosage based on body weight show only moderate fluctuations in the levels of the drug in their blood from day to day or throughout a single day.

The averages of several individual values of patients receiving sulfapyridine in a dosage by body weight vary widely from patient to patient.

In view of the several types of rather marked and unpredictable variations, it must be concluded that it is desirable to make frequent determinations of the actual level present in any given patient and subsequently to adjust the dosage according to the level desired.

URINARY TRACT, Chemotherapy in Nonspecific Infections of, Alyea, E. P., and Roberts, L. C. J. A. M. A. 115: 1345, 1940.

The sulfonamide drugs are excreted by the kidneys in a manner exactly similar to phenolsulfonphthalein.

In vitro and in vivo studies show the specificity that the sulfonamide drugs have for different bacteria and different strains of the same bacterium.

Experimental studies in vitro are not necessarily entirely comparable in vivo.

The action of sulfonamide drugs in infections of the urinary tract depends more on the tissue reaction than on direct bactericidal action in the urine.

Mandelic acid is an excellent drug for infections with colon bacilli and *Streptococcus faecalis.*

A comparison of the colon bacillus infections treated with sulfanilamide and sulfapyridine shows practically the same, or 81 per cent, cured.

A comparison of the same drugs in staphylococcic infections shows that with sulfapyridine 75 per cent were cured and with sulfanilamide 62.5 per cent.

Response to the sulfonamide drugs is rapid, usually within two or three days.

Infections complicated by other pathologic changes do not respond as favorably as the simple infections.

A comparison of sulfanilamide and mandelic acid therapy in various types of cases shows that sulfanilamide is usually preferable.

Evidence shows that the high drug concentration in the urine usually thought desirable is not necessary for cures.

A dosage of 1.7 Gm. of sulfanilamide a day with forced fluids produced as good results as 3 Gm. a day with restricted fluids. The same is true with sulfapyridine.

Many patients cannot take the large doses with restricted fluids, but the recommended small dosage is easily tolerated by all.

RENAL FUNCTION: Relative Significance of Concentration of Inorganic Sulfate in the Serum and of Its Renal Clearance, Goudsmit, A., Jr., and Keith, N. M. Arch. Int. Med. **66**: 816, 1940.

Comparative studies of blood urea, urea clearance, serum sulfate, and sulfate clearance were performed on 50 patients. The majority were suffering from diffuse arteriolar disease with hypertension of various degrees of severity; most of the remainder, from chronic glomerulonephritis and other diseases of the urinary tract. The selection of the patients was conducted in such a manner that half of the group would have normal and the other half increased concentrations of sulfate in the serum.

The increased concentration of serum sulfate was found to occur in diffuse arteriolar disease with hypertension groups 3 and 4 in 4 of every 5 cases, the urea clearance, the value for blood urea, and the sulfate clearance indicating renal damage with decreasing frequency. The correlation of renal damage sufficient to bring about actual retention of an end product of normal protein metabolism and hypersensitive vascular disease severe enough to be associated with retinitis is significant. It is assumed that arteriolar disease may well be responsible for both the retinal and the renal changes.

Generally speaking, in the more severe degrees of hypertension, as contrasted with the milder forms, there is a far greater probability of retention of either urea or sulfate in the blood than of decreases in the renal clearance of these substances. In this respect the patterns of excretion of urea and of sulfate are essentially similar. However, whereas the association of retention of urea in the blood and a normal value for urea clearance was observed in only one instance, 12 instances of increased serum sulfate in the presence of a normal value for sulfate clearance were observed.

The increase of the concentration of serum sulfate itself is held responsible for this behavior, and an analogy is seen with observations on experimental animals as well as on human volunteers, in whom the intravenous injection of sodium sulfate leads to considerable increases in the value for sulfate clearance without significantly changing renal function otherwise. In accordance with the analogy drawn from these experimental observations, the ratio of sulfate clearance to urea clearance is significantly increased in the cases of those patients who have increased values for (endogenous) sulfate in the serum as compared to those with normal values for serum sulfate.

Thus, although conclusions of prognostic significance in renal disease should not be based on the value for serum sulfate any more than on any other single laboratory index, determination of this index is a valuable adjunct in appraising renal function, and the pattern of excretion of sulfate is of considerable interest to the student of renal and vascular disease.

BLOOD SUGAR, Normal Values in Children, Rudesill, C. L., and Henderson, R. A. Am. J. Dis. Child. **61**: 108, 1941.

A total of 288 blood sugar determinations were made by the Folin-Wu method on 144 nondiabetic children from 2 to 15 years of age, inclusive. Values for both fasting and 11 A.M. blood sugar were determined on the same day and the reports were correlated.

There is no practical difference between fasting and 11 A.M. blood sugar values, since both fall within normal limits.

If the Folin-Wu method is used, normal fasting blood sugar values in nondiabetic children from 2 to 15 years of age range from 70 to 105 mg. per hundred cubic centimeters.

A review of the literature indicates that the range of normal blood sugar values in infants is probably from 60 to 100 mg. per hundred cubic centimeters in most cases.

STAPHYLOCOCCI, Technic of, The Coagulase Test for, Fisk, A. Brit. J. Exper. Path. **21**: 311, 1940.

Within wide limits the amount of sodium citrate does not materially affect the coagulase test.

Citrated plasma diluted 10 to 20 times gives satisfactory results.

Human plasma from different individuals does not show wide variations in clotting time.

The citrated plasma remains suitable for the test for several weeks if kept undiluted under sterile conditions.

Staphylococci growing in a variety of media produce coagulase. Of a number of liquid media, broth gave a culture which produced coagulation most quickly. Agar cultures may also be employed, in which case it is best to emulsify the organism in broth to form an even suspension of an opacity approximately equal to that of a twenty-four-hour broth culture.

There is an optimum ratio between amount of organism and amount of plasma.

The reaction occurs most quickly at 37° C.

The method suggested for the performance of the coagulase test is as follows:

1. Dilute citrated human plasma tenfold with normal saline.

2. Place 0.5 ml. of this diluted plasma in two small test tubes.

3. To one of these add 5 drops from a capillary pipette (approximately 0.125 ml.) of an overnight broth culture of staphylococcus or a suspension of an agar culture made in broth to an opacity approximately equal to an overnight broth culture. The second tube serves as a control and must show no clotting. Incubate the tubes at 37° C.

4. Examine the tubes after half an hour and at intervals for six hours. (Clotting of the plasma usually occurs within one hour.)

STAPHYLOCOCCI, Use of Slide Agglutination to Determine Pathogenicity of Staphylococci, Christie, R. Australian J. Exper. Biol. & M. Sc. **18**: 397, 1940.

A total of 335 strains of staphylococci of human origin and 57 strains of animal origin have been examined.

Of 269 coagulase-positive strains, 257 were agglutinated by a mixture of three antisera prepared from coagulase-positive strains; nine strains were auto-agglutinable and could not be tested. One human and two animal strains were not agglutinated; the human strain was serologically related to the coagulase-positive strains in spite of its inagglutinability with the serum mixture. Of 123 coagulase-negative strains, 120 were not agglutinated, two were auto-agglutinable, and one was agglutinated slightly.

An attempt to produce agglutinating sera with coagulase-negative strains which would agglutinate coagulase-negative and not coagulase-positive strains was unsuccessful.

HYPOPROTHROMBINEMIA, Intravenous Use of 2-Methyl-l, 4-Naphthoquinone in, Norcross, J. W., and McFarland, M. D. J. A. M. A. **115**: 2156, 1940.

2-Methyl-l, 4-naphthoquinone has been administered intravenously to 22 patients. It has proved to be efficient in raising the prothrombin level. Bile salts were not necessary for this response.

With the exception of local distress for short duration at the time of injection, no toxic manifestations have been observed.

The minimal effective intravenous dose of 2-methyl-l, 4-naphthoquinone in the adult was found to be 2 mg.

In cases uncomplicated by liver impairment a 2 mg. dose given intravenously raised the prothrombin level from 50 per cent to 80 per cent in an average of seven and one-half hours and to 100 per cent in an average of twenty hours.

A 2 mg. intravenous dose kept the prothrombin level above 90 per cent of normal for four or more days unless liver damage was present.

Severe liver damage permitted only a slight response to intravenous 2-methyl-l, 4-naphthoquinone. If liver impairment was less marked, the effect was manifested in a slower onset of response, a lessened height of response, and a shorter duration of response.

Bleeding was rapidly and effectively controlled unless severe damage was present.

The intravenous route of administration has great value in patients for whom oral administration is difficult or impossible, as well as in those in whom rapid cessation of bleeding is imperative.

PNEUMONIA in Children, Treatment of, With a Single Dose of Sulfapyridine, Platt, L. Am. J. Dis. Child. **60**: 1019, 1940.

In the children included in this study the single-dose method gave satisfactory levels in the blood in most instances, and the clinical results were as good as, if not better than, those in the group that received multiple doses. If the results are as good, it would seem desirable to adopt a single-dose method, for the following reasons:

1. It is simpler, interferes less with the child's rest and sleep during the time when he is most ill and is less demanding on the nursing staff.

2. It decreases the incidence of the most common toxic effect, nausea and vomiting.

For the ordinary case of uncomplicated pneumonia it would appear that 0.3 Gm. of sulfapyridine per kilogram of body weight administered in one dose will give satisfactory results. Lower doses are not so consistent in giving levels in the blood above 4 mg. per hundred cubic centimeters. Although the lower levels gave satisfactory results, it would seem safer to use the larger dose. If it is thought desirable to maintain levels in the blood above 4 mg. per hundred cubic centimeters for more than twenty-four hours, the dose can be repeated at eighteen- to twenty-four-hour intervals. Such repetition is probably advisable when the patient cannot be watched closely. It will probably decrease the number of cases in which a secondary rise in temperature occurs.

After the oral administration of a single dose of 0.3 Gm. of sulfapyridine per kilogram of body weight, the free sulfapyridine in the blood usually reaches levels of at least 4 mg. per hundred cubic centimeters within a few hours. In most cases a considerable concentration persists for at least twenty-four hours.

In some cases the highest levels of the blood occur after twenty-four hours, indicating that absorption from the gastrointestinal tract may continue for that length of time.

The administration of a single dose of sulfapyridine of 0.3 Gm. per kilogram of body weight to children with pneumonia gives satisfactory therapeutic results.

Levels in the blood above 2 mg. per hundred cubic centimeters give satisfactory results in most cases of pneumonia in childhood.

The use of the single dose method simplifies the treatment of pneumonia and decreases the incidence of toxic effects.

Item

Announcement will shortly be made of unassembled examinations to be given for the following positions in the West Virginia State Health Department:

Director, Hygienic Laboratory	$320-375
Senior Bacteriologist	150-200
Senior Serologist	150-200

Residence in West Virginia has been waived in consideration of the applications for these positions. However, residents of this state may be given preference in making appointments. Complete information may be obtained by writing directly to the Merit System Council, Morgantown, W. Va.